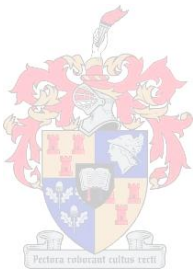
The evolution of fynbos-endemic Cephalelini leafhoppers specialising on Restionaceae

by Tobias Johannes Wiese



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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Signature:

Date: 2 / 01/ 2014

Abstract

Knowledge of the diversity and evolutionary histories of insects in South Africa's fynbos biome lags far behind what is known of the plant groups that make up this global biodiversity hotspot. To address this imbalance, I undertook a molecular phylogenetic study of fynbos-endemic Cephalelini leafhoppers that specialise on restios in the family Restionaceae. My phylogenetic results did not recover the South African Cephalelini as monophyletic, nor did I find monophyly of described species, but several monophyletic clades of species were found within Cephalelini. Furthermore, phylogenetic dating suggested that the divergence between South African and Australian Cephalelini post-dates Gondwanan vicariance, implying intercontinental extreme long distance dispersal of these insects ca. 5-11 MYA. Diversification within the Cephalelini is also much more recent than that of the Restionaceae hosts on which they specialise, negating the possibility of coevolution between plants and insects. Rather, analysis of phylogenetic conservatism of host use reveals that Cephalelini evolution has tracked the evolution of their Restionaceae hosts and that closely related insects feed on the same plant host tribes. A finer scale of tracking of host evolution (such as at the clade or genus level) is expected when taking into account how highly specific I find Cephalelini host use to be, but its absence might be explained by the recent divergence of Cephalelini relative to the age of Restionaceae. Analysis of conservatism of host use was also carried out using a phylogeny of the Restionaceae, and revealed that, overall, Cephalelini host use and avoidance have no phylogenetic bias, indicating many empty potential niches for Cephalelini, or alternately that host use is governed by factors which are phylogenetically unconstrained. Lastly, I also analysed the evolution of specialisation of Cephalelini and find no trend towards increased specialisation within the group, which is contrary to what is expected of the evolution of herbivorous insects. Overall, this study presents the first evidence of intercontinental dispersal of insect fauna between South Africa and Australia and as such highlights an unconsidered factor in the accumulation of faunal diversity in the fynbos biome. I find Cephalelini to be highly specialised in their host preference, but this pattern only becomes apparent at the tribal host level in the evolution of Cephalelini. Although Cephalelini are highly specialised, I find no evidence of evolution towards increasing specialisation within the group.

Opsomming

Ons kennis van die evolusie van fynbosinsekte is redelik beperk vergeleke met hoe veel ons weet van die plante wat die merkwaardige diversiteit van die fynbos-bioom uitmaak. In `n poging om hierdie wanbalans reg te stel het ek `n molekulêr-evolusionêre studie onderneem van die fynbos-endemiese blaarspringer-groep Cephalelini, wat op Restionaceae-gasheerplante spesialiseer. Ons het drie geen-areas geamplifiseer vir filogeniekonstruksie en dateringsdoeleindes: insek-kern H3, insek-mitokondriaal COI en insek-simbiont Sulcia 16S. Met behulp van filogenetiese analise is bevind dat die Suid-Afrikaanse Cephalelini nie `n monofiletiese groep is nie en dat beskryfde spesies ook nie monofileties is nie, maar verskeie monofiletiese spesie-groepe is wel gevind. `n Oorkruis-gevalideerde dateringsoefening dui aan dat divergensie tussen Suid-Afrikaanse en Australiaanse Cephalelini meer onlangs as die verbrokkeling van Gondwana plaasgevind het, wat impliseer dat daar uitruiling van insekte tussen die kontinente oor die afgelope 5-11 MJ plaasgevind het. Die diversiteit van Cephalelini het ook veel meer onlangs ontstaan as dié van hulle Restionacaea-gashere wat beteken dat ko-evolusie in die eng sin nie moontlik is nie. ‘n Ontleding van die evolusie van gasheer-keuse deur Cephalelini dui wel aan dat Cephalelini-evolusie die patron van hul Restionaceae-gashere volg en dat naverwante Cephalelini dieselfde stam van Restionaceae as gashere verkies. Die bevinding dat die Cephalelini hoogs gespesialiseerd is in hulle gasheer-keuses lei ‘n mens tot die verwagting dat die evolusie van Cephalelini meer getrou die evolusie van hulle gashere sal volg as wat wel die geval is (dalk op die vlak van groep of genus), maar die relatiewe jeugdigheid van die Cephalelini vergeleke met die ouderdom van die Restionaceae is moontlik die rede hiervoor. Analise van gasheer-keuse is ook uitgevoer op `n filogenie van die Restionaceae wat aantoon dat daar nie ‘n beperking van verwantskappe is tussen gashere wat verkies of verwerp word nie, wat daarop dui dat daar `n menigte nisse is wat oënskynlik onbenut is, of dat Cephalelini gasheer-keuse bepaal word deur `n Restionaceae-eienskappe wat nie filogeneties beperk is nie. Laastens het ek die evolusie van spesialisering deur Cephalelini ontleed, maar geen neiging tot toenemende spesialisering binne die groep gevind nie, wat onverwags is in die lig van vorige studies. As `n geheel bied hierdie studie die eerste bewys van die uitruiling van insek-fauna tussen die Suid-Afrikaanse fynbos-bioom en ander kontinente, wat `n onopgetekende invloed op die diversiteit van diere in die fynbos is. Cephalelini is `n hoogs gespesialiseerde groep insekte, maar uit `n filogenetiese perspektief word dit eers waargeneem op die vlak van gasheerstam. Ten spyte van hulle hoë vlak van spesialisering het ek geen bewys gevind van `n toename daarin binne die evolusie van die groep nie.

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I thank my mothers' spirit of persistence and curiosity for helping me to embark on this scholarly expedition and seeing it through and I thank my fathers' ideals of accuracy and thoroughness (whilst still telling a good story) to which I eventually returned for the completion of this project.

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This work has been presented in part at two conferences:

SASSBX- The South African Society for Systematic Biology 10th meeting, 2012

and

Fynbos Forum 2013 – Celebrating Fynbos in a Centenary year

Dedications

I dedicate this thesis to the myriad distractions that emerge from the ether whenever a human has to sit in front of a computer for months: to recurve self-bows, high-lining, slack-lining, sunrise from a cave, cave-painting, engraving, tattoos, short film, The Mamas and The Papas, The Animals, woodwork, chess, feather-collecting, bird-watching, flint-knapping, Swimming Dragon, handstands, high dives, tall women with long hair, short women with short hair, strong women and mountains, baking bread, chasing rabbits, sunshine, Elvis and to Jack.

"A human being should be able to change a diaper, plan an invasion, butcher a hog, conn a ship, design a building, write a sonnet, balance accounts, build a wall, set a bone, comfort the dying, take orders, give orders, cooperate, act alone, solve equations, analyze a new problem, pitch manure, program a computer, cook a tasty meal, fight efficiently, die gallantly. Specialization is for insects."

Robert Heinlein, author

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to the right of clades as in Fig. 3.1A). The vertical dashed line delineates the two Restionaceae tribes (as in Fig. 3.1A), Restioneae on the left and Wildenowieae on the right. Numbers in boxes on left and right of the vertical dashed line indicate the proportion of encountered hosts used in the Restioneae and Wildenowieae tribes respectively for each of the three insect clades. Significant difference in proportions found by Z-test for Clade C2 ($z = -3.34$, $p < 0.0005$). Sample sizes were too small to test Clade D and C1 (Sprinthall, 2002). Black dots on Restionaceae phylogeny indicate well-supported nodes (posterior probability > 0.91, Zander *et al.*, 2004).

Figure 3.2 Evolution of host preference on the Restionaceae phylogeny (consistently on the left) and Cephalelini phylogeny (consistently on the right) with posterior probabilities of supported nodes indicated. **A** The phylogram on the left is a Bayesian inference phylogeny of the Restionaceae pruned to only include restio species preferred by each Cephalelini species. The colours orange and yellow at the tips of the tree indicate the Restioneae and Wildenowieae tribes (Briggs & Linder, 2009) respectively. The tree on the right is a Bayesian inference phylogeny of Cephalelini species in the genera *Duospina* and *Cephalelus*, based on *H3* and *CO1*. The colours blue, red and green indicate a *Duospina* clade, D, and two reciprocally monophyletic clades within *Cephalelus*, C1 and C2, respectively. **B** Same trees as in A with lines indicating the preferred host associations of each Cephalelini species. Line colours correspond to colours of Cephalelini clades in A. **C** Phylogram of the Restionaceae showing host preference of different Cephalelini clades (as in A), with significant phylogenetic similarity found between hosts preferred by different Cephalelini clades ($\lambda = 0.66$, $p<0.05$). **D** Cephalelin phylogram showing significant phylogenetic signal of preference for host tribes by Cephalelini species ($\lambda = 1$, $D=-1.1$)

Figure 3.3 Illustration of host preference determination for 11 of the 18 species of Cephalelini. The remaining 8 had 2 or less hosts in their host range and are not presented here. Bars represent the percentage of the total abundance of a Cephalelini species added by considering densities on each additional host. Hosts are ranked from those on which a Cephalelini species is most abundant to least abundant. Dark grey bars indicate the preferred hosts that account for the first 50% of the cumulative density.

Figure S2.1 Chronogram of Cephalelini (top clade, in red) and the outgroups used for dating (bottom clade, in blue) based on *CO1*. Median age estimates from Bayesian inference using a priori rate calibration of *CO1* indicated at nodes.

Figure S2.2 Chronogram of Cephalelini (in red) and the outgroups used for dating (in blue) based on *Sulcia* 16S rDNA. Median age estimates from Bayesian inference using *Sulcia* host fossil calibrations from Moran *et al.* (2005) are indicated at nodes.

Chapter 1

Introduction

Surprisingly little knowledge exists on insect diversity and the processes that underlie it in South Africa's hyper-diverse Cape Floristic Region (CFR, Goldblatt & Manning, 2002). Since plant and insect diversity appear to be causally linked (Ehrlich & Raven, 1964; Huston, 1979), this lack of knowledge represents a significant gap in our understanding of how different trophic levels affect each other's diversification in the CFR. This thesis is an investigation into the evolution of CFR insects, focusing on a tribe of leafhoppers, the Cephalelini, that specialise exclusively on Restionaceae host plants. In the second chapter I address Cephalelini taxonomy and diversification by reconstructing a molecular phylogeny for the group. I also estimate ages of evolutionary events implied by the phylogenetic tree to test for temporal congruence of Cephalelini evolution with expected drivers of insect diversification. In the third chapter I investigate the evolution of host use of South African Cephalelini and ask whether these leafhoppers have evolved to prefer certain clades of Restionaceae hosts, whether closely-related Cephalelini prefer closely-related hosts and whether the different hosts that particular leafhoppers utilize are closely-related. Lastly, I test for a trend towards increased specialisation within Cephalelini.

Molecular phylogenetics uses statistical analysis of gene sequences to infer the evolutionary history of a group of related organisms (Huelsenbeck *et al.*, 2001) and can provide detailed information on taxonomy (Felsenstein, 2004). The sheer number of characters that are available from gene sequences allows phylogenetic insights into the evolution of species beyond that available from classical morphological cladistic approaches (Hillis et al., 1987).

The nature of nucleotide sequence evolution also means that the amount of sequence divergence between species can be correlated with the amount of time that has elapsed since their divergence (Zuckerkandl & Pauling, 1962), and with appropriate calibration can provide realistic age estimates of speciation events (Drummond *et al.*, 2006). In lieu of fossils, rate-calibration can be used to estimate node ages within a phylogeny. Some genes, such as mitochondrial genes in insects, have been used in enough fossil-calibrated studies of genetic evolution that a confident estimate has been made of the typical rate of sequence evolution of mitochondrial insect genes (Papadopoulou *et al.*, 2010). Fossil calibration of phylogenies is more reliable than rate calibration, because fossils provide calibration that is specific to the group under study (Near *et al*., 2004), an important feature when considering that evolutionary rates of the same gene can vary between related groups of organisms (Thomas *et al.*,

2006). Nevertheless, either method provides valuable information on the chronology of evolutionary events not obtainable by other means. A third method of phylogeny calibration available to insect evolutionary studies is worth mentioning here. All sap-feeding insects harbor intracellular nutritional endosymbionts with whom they are locked in a obligate, biosynthetically complementary relationship, each providing the other with nutrients they cannot themselves synthesise (Moran *et al.*, 2005). The association is ancient (>260MY) and the molecular evolution of insect bacterial symbionts has been well-characterised and dated using several fossil calibrations of their hosts (Moran *et al.*, 2005). Therefore, if symbiont gene sequences can be extracted from an insect host, then these sequences provide a calibration of host evolution that is useful when insect fossils are lacking or uninformative (Dietrich *et al.*, 2001).

Molecular dating has been very useful in determining historical landscape effects on the evolutionary history of species, as it provides a time-stamp on evolutionary events that can be compared with dated geological events, such as the formation of mountain ranges and land-bridges, island formation, climatic changes and the movement of continental landmasses (Morrone *et al.*, 1995). Whilst the importance of geographic effects on biological evolution are well-recognised (Wiley, 1988), it is not the only factor to consider. For example, the contemporary distributions of Gondwanan species are often assumed to be the result of a common ancestor that was distributed across Gondwana before the separation of its constituent landmasses (Waters *et al.*, 2013), but distribution information alone cannot confirm this hypothesis. Divergence times and a species' dispersal ability also need to be taken into account to support explanations of contemporary species distributions (Gillespie *et al.*, 2013), which until relatively recently has not been done by biogeographers (Waters *et al.*, 2013). Dated molecular studies indicate many groups with a Gondwanan distribution have diverged more recently than Gondwanan vicariance, implying long-distance dispersal in their evolutionary histories (CFR examples from: Linder *et al.*, 2003, Johnson & Briggs, 1975, Bergh *et al.*, 2009; Leese *et al.*, 2010; Nikula *et al.*, 2013; Townsend *et al.*, 2011; Tolley *et al.*, 2013; all plants) and illustrates the importance of using all available information to test an hypothesis.

Another field that has been similarly changed by the evidence provided by molecular dating is the study of coevolution. Without the time-stamp provided by the dating of phylogenies of interacting and potentially coevolving groups, the entire temporal dimension of coevolution is ignored, a pivotal assumption of the process (Janzen, 1980). Coevolving organisms or groups of organisms are instrumental in each other's diversification, i.e. they exert reciprocal evolutionary pressures on each other (Ehrlich & Raven, 1964), which means that their diversification overlaps in time when they are truly coevolving. As the coevolutionary process continues over millennia, it can generate similar diversification patterns in the evolutionary histories of interacting species, and as a result topological congruence between the phylogenies of interacting groups is taken as evidence of coevolution. However, it was recognised early on that topological congruence alone is not enough to prove

coevolution from a phylogenetic perspective and that the timing of corresponding divergence events needs to be taken into account, because congruent topologies can arise due to a process termed sequential evolution (Jermy, 1976), whereby the evolution of one group of organisms tracks an evolutionarily conserved trait in another group, which is why sequential evolution is also sometimes referred to as phylogenetic tracking (Menken, 1996; Percy *et al.*, 2004). The distinction between coevolution and phylogenetic tracking is important when trying to identify important factors in the genesis and maintenance of species diversity, in order not to overestimate the symmetry of biotic influences on diversification.

Coevolution also has important ecological implications. For example, the coevolutionary biochemical arms race between plants and insects is considered the main driver of increased specialisation by phytophagous insects (Jaenike, 1990), due to the fact that when insects evolve the ability to overcome the defenses of a specific plant, this usually comes at the cost of being unable to feed on a broader range of plants (Futuyma & Moreno, 1988). When one considers the number of hosts that a species of insect can feed on as an indicator of the width of its niche (Roughgarden, 1972), and you consider that increased specialisation therefore decreases niche width, it logically follows that any group with a tendency towards specialisation is subdividing available niche space into smaller and smaller portions. The fact that herbivorous insects have a tendency towards increased specialisation (Nosil, 2002; Mayhew, 2007) then goes a long way toward explaining their disproportionate diversity relative to other groups of insects less prone to specialisation (Mitter *et al.*, 1988). However, considering chemical diversity of hosts as the only driver of specialisation and diversification within plant-insect interactions overlooks other potentially important factors, such as the availability of enemy-free space. For example, insects might be limited to colonisation of hosts on which they are morphologically similar enough to avoid predator detection (Van Valen, 1965). Determining the factors in a plant-insect interaction that have been influential in driving diversification is made possible by several different analytical techniques (Pagel, 1999; Blomberg *et al.*, 2003; Fritz & Purvis, 2010) that allows one to test the degree of phylogenetic conservatism of a trait in the evolution of a group of organisms. Identifying similarly conserved traits in the evolution of co-diversifying groups then points to factors that have likely been instrumental in the progression of coevolution.

Dated molecular phylogenies have been constructed for a large number of the endemic plant groups that make up part of the more than 9000 species (Linder, 2003) found in the hyperdiverse CFR (Cape Floristic Region; Goldblatt & Manning, 2002)(e.g. Linder *et al.*, 2005 – Restionaceae; Pirie *et al.*, 2012 – Erica; Hoot *et al.*, 1998 – Proteaceae; Bakker *et al.*, 2004 – Pelargonium; Van der Niet *et al.*, 2005; Goldblatt *et al.*, 2002 – Moraea; Forest *et al.*, 2007 - *Muraltia*). The availability of these dated phylogenies in conjunction with information on the timing of geological and climatic change in the CFR has resulted in many tests of the influence of historic abiotic factors on the evolution of plant diversity (Van der Niet *et al.*, 2005; Cowling, 2009; Verboom *et al.*, 2009). The wealth of such

studies has allowed generalization about the drivers of diversification that act across many different taxa (Linder, 2003), providing insight into factors that are important in generating the incredible diversity of CFR plants (Linder, 2005; Linder & Hardy, 2005). CFR endemic insects have not enjoyed the same degree of attention as the plants, both overall and in a molecular phylogenetic context (but see Price *et al.*, 2007 & Price *et al.*, 2010 – cicadas; Sole *et al.*, 2013 – lacewings; Damgaard *et al.*, 2008 – heelwalkers), which means that no general historical driver of insect diversity in the CFR can as yet be identified, nor have phylogenetic estimates of coevolution with the hyperdiverse flora been possible.

The Cephalelini are a tribe of leafhoppers that comprise two described genera that occur exclusively on Restionaceae host plants (Davies, 1988), a characteristic element of the South African fynbos flora. Cephalelini and Restionaceae have a typical Gondwanan distribution, with relatives in Australia and New Zealand, in addition to South Africa. Cephalelini species exhibit highly specialised host use (Augustyn et al., 2013) and appear to be morphologically adapted to resemble their Restionaceae hosts (Davies, 1988, personal observation), ostensibly to avoid detection by predators. Both of these factors indicate a potential link between the evolutionary histories of the groups and suggest the groups as a good model system for investigating plant-insect coevolutionary dynamics in the CFR.

The diversification history of Cephalelini is largely unknown, since the only attempt at phylogeny reconstruction was based on morphological characters and received very weak support (Prendini et al., 1998). On the other hand, fossil-dated molecular analysis of the Restionaceae hosts of Cephalelini have shown these plants to be ancient additions to the CFR (Linder & Hardy, 2005), suggesting that the Cephalelini might also be

In this thesis I estimate the ages of Cephalelini evolutionary events by generating a rate and fossil-calibrated, multi-gene phylogeny of the group (Chapter 2). I ask what these ages indicate about the influence of Gondwanan vicariance on the current distribution of Cephalelini, the possibility of coevolution between Cephalelini and their Restionaceae hosts and whether Cephalelini diversification has been influenced by some of the same broad, historical factors suggested to have influenced the evolution of the Cape flora. I also investigate the evolution of host use of South African Cephalelini from a phylogenetic perspective using multiple analytical techniques (Chapter 3). I ask whether Cephalelini have evolved to prefer certain clades of Restionaceae hosts, whether closely-related Cephalelini prefer closely-related hosts and whether the different Restionaceae hosts that a Cephalelini species uses are closely-related. Here I also test for a trend towards increased specialisation within Cephalelini. Overall, the results of this study provide novel insights into the evolutionary history of fynbos-specialist insects as well as identifying a novel influence on the accumulation of faunal diversity in the biome.

Chapter 2

Recent colonization and radiation of specialist Cephalelini leafhoppers on Restionaceae in the Cape Floristic Region

Abstract South Africa's Cape Floristic Region (CFR) is one of the world's richest biodiversity hotspots. The dynamics underlying CFR biodiversity have become increasingly well understood for the plants of the region, but surprisingly little is known of the evolutionary histories of the insects that utilise these plants. The leafhopper tribe Cephalelini has a Gondwanan distribution, with representatives in South Africa, Australia and New Zealand. Two genera, *Cephalelus* and *Duospina*, are endemic to the CFR and specialise exclusively on Restionaceae host plants. I reconstructed a dated molecular phylogeny for the group based on three gene regions: mitochondrial *CO1*, nuclear *H3* and *Sulcia* endosymbiont 16S rDNA sequences, in order to determine the age of divergence between South African and Australian Cephalelini as well as investigate the possibility of coevolution between Cephalelini and their Restionaceae hosts. I carried out a dating exercise which was validated by using two independent calibration strategies, the first of which used the range of known rates of insect mitochondrial evolution from >30 studies and the second of which used five host-fossil calibrations of *Sulcia* 16S rDNA evolution from a previous study to estimate node ages. I found geographical monophyly for both South African and Australian Cephalelini clades, but little species-level monophyly within Cephalelini. The dating analysis suggests that South African and Australian Cephalelini leafhoppers diverged less than 11 MYA, implying that extreme long distance (intercontinental) dispersal must have occurred within the group. This young age also implies that Cephalelini could not have coevolved with their Restionaceae hosts (most recent diversification ~13 MYA), nor been subject to the same climatic drivers of diversification that have been important to CFR plant lineages. I conclude that transoceanic dispersal is the most parsimonious explanation for the current distribution of Cephalelini and that this points to a hitherto unconsidered effect on insect faunal assemblages in the CFR.

Introduction

Since publication of Thunbergs' *Flora Capensis* in the early 1800's (Thunberg, 1807), we have gained considerable understanding of the diversity (Kruger & Taylor, 1980; Goldblatt, 1997; Goldblatt & Manning, 2002) and diversification (Linder, 2003; Cowling *et al.*, 2009, Schnitzler *et al.*, 2012) of the hyper-diverse flora of the Cape Floristic Region (CFR, Goldblatt & Manning, 2002). Recent developments in molecular phylogenetic tools have provided much insight into the evolution of the CFR flora (Linder, 2005; Barraclough, 2006; Verboom *et al.*, 2009, Schnitzler *et al.*, 2011). In contrast, the diversity and diversification of insects of the region has received relatively little attention (broad work by Giliomee 2003 & Proches *et al.* 2009), with only a few recent studies focusing on insect molecular phylogenetics (Price *et al.*, 2011- Dirini butterflies; Price *et al.*, 2007 & Price *et al.*, 2010 – cicadas; Sole *et al.*, 2013 – lacewings; Damgaard *et al.*, 2008 – heelwalkers, Ware *et al.*, 2009 – dragonflies, Pitzalis *et al.*, 2010 – blister beetles). Importantly, to my knowledge, no studies have focused on insects endemic to the CFR or specialising on CFR endemic plant lineages.

Studies on the evolutionary history of CFR insects may allow comparisons to the extensive biogeographic and climatic scenarios reconstructed around the diversification histories of plants in the region (Cowling *et al.*, 2009; Van Der Niet & Johnson, 2009; Verboom *et al.*, 2009), providing insight into whether plants and insects experience similar evolutionary pressures in this biodiversity hotspot. The largest and most obvious biogeographical effect on CFR insect evolution to test is that of Gondwanan vicariance, as has been done for a number of plant lineages within the CFR that have a Gondwanan distribution, such as the typical fynbos families of Restionaceae (Linder *et al.*, 2003) and Proteaceae (Johnson & Briggs, 1975). Despite their Gondwanan distribution however, fossil-dated molecular analyses indicate sister group relationships within these families across continents that post-date Gondwanan break-up, pointing toward long-distance, intercontinental, transoceanic dispersal in their evolutionary histories (Restionaceae - Linder *et al.*, 2003; Proteaceae – Barker *et al.*, 2007). Whether the timing of the dispersal of Gondwanan insect lineages follows the same patterns remain to be tested.

Plant and insect diversification histories can also be linked when interacting groups exert reciprocal evolutionary pressures on each other and such groups are then said to coevolve (Ehrlich & Raven, 1964). However, until the incorporation of molecular phylogenetic dating into studies of coevolution, an important aspect of the process remained untested; namely, the timing of diversification within interacting groups. Fossil- or rate-calibrated phylogenies provide ages of diversification events, a crucial component for distinguishing between true coevolution (termed *reciprocal diversification* by Janzen 1980) and phylogenetic tracking (termed *sequential evolution* by Jermy 1976). In the case of coevolution, diversification in two groups has to be temporally overlapping as well as the result of reciprocal selective pressures. In the case of phylogenetic tracking, diversification in one group tracks

evolutionarily conserved features in another group. The phylogenetic signal of sequential evolution by phylogenetic tracking can appear similar to patterns of coevolution, but selective pressures are non-reciprocal and diversification can occur at separate times in the evolution of the two groups. Examination of dated phylogenies of interacting groups has shown that coevolution between groups of species is rare, with sequential evolution being the rule (Mitter *et al.*, 1991; Menken, 1996; Percy *et al.*, 2004).

While only a few studies have investigated plant-herbivore interactions in the Cape, several potential cases of coevolutionary interactions have been proposed. Leafhoppers in the tribe Cephalelini (hereafter restiohoppers) (Hemiptera: Cicadellidae) feeding on Restionaceae (hereafter restios) is one such example (Davies, 1988). Restiohoppers are morphologically cryptic on restios (Davies, 1988) as well as highly specialised in their host choice (Augystyn *et al.*, 2013), leading to an expectation of possible coevolution between the two groups (Prendini *et al.*, 1997). In order to better understand potential coevolutionary dynamics between plants and insects within the hyperdiverse CFR (Goldblatt & Manning, 2002), I use the dated molecular phylogeny of restios (Linder *et al.*, 2005) in conjunction with a dated molecular phylogeny for restiohoppers to determine whether diversification within plant and insect groups were historically contemporaneous (i.e. whether they could have coevolved). Since both restios and restiohoppers have a Gondwanan distribution, I also test whether restiohopper evolution bears a signal of Gondwanan vicariance. Lastly, I test whether an implied driver of the diversification of several CFR plant lineages (including Restionaceae, Cowling *et al.*, 2009), namely the onset of climatic changes at the Eocene-Oligocene boundary and the continuation of climatic changes up to the Miocene-Pliocene boundary, could have influenced the diversification of restiohoppers.

Specifically, using a dated phylogeny for restiohoppers I aim to address the following questions:

1) does divergence between South African and Australian Cephalelini correspond with the timing of the breakup of Gondwana?

2) has the diversification of Cephalelini been contemporaneous with that of their Restionaceae hosts, i.e. could they have coevolved?

3) does the timing of diversification of Cephalelini in the CFR correspond with Cenozoic climate changes?

Methods

Study system

South African Cephalelini (Hemiptera: Cicadellidae) contains 21 described species in two genera, *Cephalelus* (18 species) and *Duospina* (three species) that occur exclusively on Restionaceae throughout the Cape Floristic Region (Davies, 1988; Prendini *et al.*, 1998). They have a specially adapted morphology that varies between species and appears to match the morphology of the dried leaf sheaths of their Restionaceae hosts (Davies 1988). South African Cephalelini are also highly specialised in their host choice at the population level (Augustyn *et al.*, 2013), which raises the possibility that they could have coevolved with their Restionaceae hosts.

Cephalelini, like all leafhoppers, are plant-sap feeders. Since plant-sap is a nutritionally deficient diet for eukaryotic metabolism (Douglas 2008), sap-feeding insects house primary endosymbiotic bacteria that synthesise necessary amino acids (Moran *et al.*, 1998). *Sulcia* bacteria, in particular, are known to be the primary nutritional symbionts of the majority of sap-feeding Auchenorrynchan species that have been investigated (Moran *et al.*, 2005). Thus I expected that Cephalelini should house *Sulcia* endosymbionts.

Both Cephalelini and Restionaceae species co-occur in South Africa, Australia and New Zealand, all of which once formed part of the supercontinent Gondwana. A previously dated phylogeny suggested that the dispersal of Restionaceae occurred from Australia to South Africa (Linder *et al.*, 2005) and, more importantly, that their dispersal is more recent than the breakup of the supercontinent Gondwana (Linder *et al.*, 2003).

Sampling

South African Cephalelini were collected between March 2010 and April 2013. Collection of insects was done by vacuum-suctioning of Restionaceae host plants using a leaf blower modified by the addition of a fine mesh bag to the front of the intake tube. Captured specimens were removed from the mesh bag and killed by submersion in 96% ethanol and kept at -4°C until DNA was extracted. Sampling was carried out at more than 70 sites across the South African fynbos to cover the known range of restiohoppers as described in Davies (1988). GPS coordinates of collection localities are included in Table 2.1.

All captured Cephalelini specimens were identified to the species level by dissection of genitalia and comparison with species descriptions and illustrations by Davies (1988). Voucher specimens of insects that underwent non-destructive DNA extraction consist of the entire exoskeleton plus the genitalia and of only the genitals for those that were ground up for DNA extraction. Vouchers are

housed in the Botany & Zoology Department (AG Ellis collection), Natural Sciences Building, Stellenbosch University.

An outgroup Cephalelini species from Australia was chosen in order to date the age of divergence between South African and Australian Cephalelini. The outgroup Cephalelini species *Linacephalus foveolatus,* from Barrow Island off the north-western shore of Australia, was obtained from the collections of the Department of Environment and Agriculture of the Curtin University of Technology.

DNA extraction, gene amplification, DNA sequencing and sequence alignment

DNA of all collected specimens was extracted using a Qiagen DNEasy Blood and Tissue extraction kit (QIAGEN, supplied by Whitehead Scientific, Cape Town) following the manufacturer's protocol. Polymerase Chain Reactions (PCRs) were carried out with two primer pairs (Table 2.2) in order to amplify one insect mitochondrial (*CO1*) and one nuclear (*H3*) gene region. Additionally, I also screened all Cephalelini species for the presence of *Sulcia* spp. primary endosymbiotic bacteria, using a *Sulcia*-16S rDNA-specific primer pair (Table 2.2). PCR cycles for *CO1* and 16S rDNA genes were: 5 min initial denaturation at 95 °C, followed by 30 cycles of 1 min at 94 °C (denaturation), 1 min at the region-specific annealing temperature (Table 2.2) and 1 min at 72 °C (elongation). This was followed by a 10 min final elongation step at 72 °C. I used the same cycle for the *H3* gene amplification but with a lowered elongation temperature of 65 °C. PCR products were visualised on an agarose gel, purified using a QIAGEN DNA Purification Kit (QIAGEN, supplied by Whitehead Scientific, Cape Town) and spectrophotometrically analysed using a NANO6000 spectrophotometer to determine the DNA concentration and purity of each sample. For samples of suitable concentration and purity (>10ng and 260/280 ratio between 1.6 and 2), automated sequencing was carried out in a single direction for nuclear and mitochondrial amplicons and in both directions for the symbiont 16S rDNA amplicons using standard BigDye chemistry and an ABI sequencer at Macrogen (Macrogen, South Korea) or at Stellenbosch University's Central Analytical Facility.

Raw sequences were edited in BioEdit v7.0.9.0 (Hall, 1999). Clean sequences were aligned using MAFFT Online (Katoh *et al.*, 2002). Each gene region was analysed separately and a dataset consisting of concatenated *CO1* and *H3* sequences was also analysed.

Phylogenetic Analysis

Each fully-aligned dataset was analysed using jModelTest v.2.1.3 (Posada, 2008) to determine the most appropriate substitution model for phylogeny reconstruction using the Akaike Information Criterion and Decision Theoretic (DT) scores retrieved from the analyses. The AIC approach finds the

best model with the least parameters by penalising overparameterisation and the DT approach selects for models to minimise the error of estimating branch lengths (Lemey *et al.*, 2009).

Information on aligned sequences as well as the substitution schemes and clock models used for the different partitions can be found in Table 2.4. Where model choice differed between AICc and DT approaches, both models were used in phylogenetic analysis, but because no significant difference between tree likelihoods was found (results not shown) only DT model choice is reported here.

BEAUTi (Drummond and Rambaut, 2007) was used as a convenient graphical interface for generating inputs for phylogeny estimation by Bayesian inference in BEAST (Drummond and Rambaut, 2007). For each of the datasets, three BEAST analyses were carried out, each consisting of 10 000 000 generations with sampling at every 1000^{th} generation. Results from the three different analyses for each dataset was combined (using logCombiner; Drummond and Rambaut, 2007) to ensure that results were not being biased by the effect of a single random starting condition. BEAST log files were analysed in TRACER (Drummond & Rambaut, 2007) to determine whether convergence on a likely topology had occurred and whether effective sample sizes of the different sampled priors were large enough (>200) to rule out the possibility of autocorrelation among data points used to estimate posterior probabilities. The presence or otherwise of a molecular clock was tested in two ways. First, a Maximum Likelihood test of a molecular clock was performed in Mega v. 5.1 (Tamura *et al.*, 2011). Second, the BEAST output analysed in TRACER gives two statistics of relevance to the presence or absence of a molecular clock in the data. The Coefficient of Variation statistic must be significantly different from 0 and the ucld.stdev statistic must be significantly higher than 1 in order to reject the molecular clock in a dataset. The former indicates whether significant among-branch rate heterogeneity exists and the latter indicates whether the standard deviation of branch rates is greater than the mean rate (Lemey *et al.*, 2009). If these statistics are ~0 and <1 respectively, then sequence evolution is clock-like.

To determine whether *CO1* and *H3* could be combined into a single phylogenetic analysis, I performed an incongruence length difference test (ILD test, Farris *et al.*, 1995) in R (script available on request). The test consists of first establishing the observed difference in the number of parsimony steps between a tree based on the combined data and the sum of two trees based on the individual datasets (combined-(indvidual$_1$ + individual$_2$), which is termed the incongruence length difference (ILD, Farris *et al.*, 1995). Significance of the observed ILD statistic is then determined by randomisation. Characters within each partition are randomly reshuffled between partitions (with the original size of each partition retained) and the ILD calculated each time, providing a null distribution against which to test the observed ILD. If the observed ILD is larger than the expected ILD, the phylogeny of the combined dataset is less parsimonious than the phylogenies of its parts, indicating that concatenation is impractical (Farris *et al.*, 1995).

ILD testing indicated that *CO1* and *H3* were not incongruent (ILD: $p = 0.3$), allowing the two gene regions to be combined into a single analysis. Accessions of *CO1* and *H3* were not necessarily available for the same individuals; therefore I created sequences consisting entirely of missing data in these cases in order to be able to concatenate *CO1* and *H3* for a combined analysis. Concatenation with *Sulcia* 16S rDNA was not attempted, due to the small size of the dataset (only 13 successful sequences for nine different species, Table 2.1) and the lack of divergence between symbiont sequences of closely-related Cephalelini. I analysed four datasets for topology estimation: a *CO1*-only dataset, an *H3*-only dataset, a combined *CO1* and *H3* dataset as well as a 16S rDNA dataset. I also analysed a further three datasets for the dating exercise described below: *CO1* plus Cicadellid outgroups, *H3* plus Cicadellid outgroups and 16S rDNA plus Cicadellid outgroups.

Rates of molecular evolution

No closely-related fossils are available for calibration of the Cephalelini phylogeny; therefore I approach in-group diversification dating in two alternative ways.

1) Insect mitochondrial *CO1* substitution rates: I estimated node ages within the Cephalelini tree using overall rates of mitochondrial molecular evolution for insects (1-2.3% divergence per MY; Papadopoulou *et al.*, 2010), corresponding to 1.75 - 4.04 substitutions per MY for this dataset. I chose the entire range rather than a single rate in order to account for variation in known rates of sequence evolution. This range was specified in BEAST by setting a uniform prior on the mean rate of sequence evolution, with upper and lower bounds corresponding to the highest and lowest substitution rates for *CO1* mentioned above. Additionally, *CO1* calibration was used to estimate the evolutionary rates of *H3* and 16S rDNA (with precedent from Rix & Harvey, 2012 and Moran *et al.*, 1993 respectively), because the rate of mitochondrial evolution of insects is much better characterised than that of nuclear and symbiont genes (Papadopoulou *et al.*, 2010). First, a tree was generated using only *CO1* data and a specified prior rate range of 1-2.3% sequence evolution per million years. The estimated root node age of the *CO1* tree was then used as a calibration point for the *H3*-only and *Sulcia* 16S rDNA-only phylogenies to estimate their respective evolutionary rates. The root nodes in all three analyses were generated by using sequences from three outgroup Cicadellid (leafhopper) species (Table 2.3, Fig. S1). These distant outgroups were chosen to avoid confounding divergence estimates from a node calibration that is too near the ingroup and to have fully comparable datasets across all genes, because sequences for the Australian outgroup species could not be retrieved for all three genes used in this study.

2) *Sulcia* 16S rDNA rates: I also estimated a node age of South African Cephalelini using the *Sulcia* 16S rDNA dataset and previously published sequences which provide five fossil-dated nodes ranging

from 23-260 MYA (Moran *et al.*, 2005). These ancient fossil calibrations are possible with *Sulcia* due to the comparatively slow rate of *Sulcia* evolution (Moran *et al.*, 1993), which overcomes problems associated with long-branch attraction (Bergsten, 2005).

Evolutionary rates and root node ages were compared by extracting the post burn-in values of these parameters from the BEAST log files of the relevant analyses. Overall, this approach should allow for a more robust assessment of evolutionary rate estimation and node age estimates of the different genes than using only one calibration across all genes.

Results

Sampling

We sampled 17 of the 21 Cephalelini species described by Davies (1988) and Prendini (1997) and three additional species which, on the basis of genital morphology, appear to be new to science. I refer to the first undescribed species as *Cephalelus sp. nov.*, because its external morphology and genitalia resemble that of the genus *Cephalelus*. The remaining two species I designate *Cephalelini* s*pp.1.* and *Cephalelini* s*pp.2.*, because their corkscrewing genitalic styles more closely resemble those of the Australian Cephalelini than the evenly-shaped styles of the South African genera, *Cephalelus* and *Duospina*.

DNA sequencing yielded 43 *CO1* amplicons, 46 *H3* amplicons and 13 *Sulcia* 16S rDNA amplicons (Table 2.1). Aligned *CO1* sequences were 351 basepairs long, with 128 variable sites; aligned *H3* sequences were 268 basepairs long, with 80 variable sites and aligned *Sulcia* 16S sequences were 1037 basepairs long, with six variable sites. Four insertions/deletions were present in the *Sulcia* 16S rDNA dataset. These indels were incorporated into phylogenetic analysis by creating a new alignment by hand that scored insertion/deletion events as equally likely and added as an additional partition to the 16S rDNA analysis. No insertions/deletions were found in the *CO1* or *H3* datasets.

Phylogeny

The South African Cephalelini cannot confidently be said to be either monophyletic or paraphyletic, due to lack of support at deeper nodes, but rather several monophyletic clades are found within Cephalelini that are as a whole polytomous at the base of the phylogeny (Fig 2.1). The genus *Cephalelus* is probably rendered paraphyletic by the genus *Duospina*, with some support (>0.8, Fig. 2.1) for a *Cephalelus-Duospina* clade that excludes *C. cygnastylus*. However, this potential paraphyly is supported only by analysis of *CO1*, because I was unable to retrieve nuclear *H3* sequences for this species. Geographic monophyly is recovered for South African Cephalelini clades with respect to the

Australian Cephalelini species *Linacephalus foveolatus*. I found little mid- to low-level support in the tree, with nodes older than 1.5 MY collapsing to a polytomy at the base (following Zander *et al.*'s, (2004) significance cut-off for posterior probabilities of 0.91). Six distinct clades were retrieved (Fig. 2.1) within the currently described genera: clade *a*, which contains nine *Cephalelus* species*;* clade *b*, which contains four *Cephalelus* species as well as *C. spp. nov.* and clade *c*, which contains the three *Duospina* species. Clade *d* contains *C. cygnastylus*, clade *e* contains *Cephalelini spp. nov.* 1 and clade *f* contains *Cephalelini spp. nov. 2*. I only show the concatenated tree, because the individual topologies of *CO1* and *H3* were not conflicting.

Rates of molecular evolution

1) *CO1* rates and dates: calibration of the rate prior range to 1 – 2.3% divergence per MY (Papadopoulou *et al.*, 2010) for *CO1* evolution recovered the time to the most recent common ancestor (TMRCA) of South African Cephalelini and the Cicadellid outgroups as 62.29 MYA (95% highest posterior density (HPD) = 32.1 – 124 MY; Fig. S1). Root node age calibration of the *H3* tree using this date delivered a median rate divergence for *H3* of 0.11%/MY (95% HPD = 0.075% - 0.15%/MY). The same calibration of the *Sulcia* 16S rDNA tree resulted in a median rate of sequence evolution of 0.093%/MY (95% HPD = 0.061% - 0.14%/MY).

2) *Sulcia* 16S rDNA rates and dates: combining our Cephalelini 16S rDNA sequences with those from Moran *et al.*, (2005) and using five fossil calibrations resulted in a median rate of *Sulcia* 16S rDNA sequence evolution of 0.054% divergence/MY (95% HPD = 0.035%-0.085%/MY) and a TMRCA of South African Cephalelini and outgroup Cicadellids of 107.2 MY (95% HPD = 63.7-160.8MY; Fig. S2). Root node age calibration of the *CO1* tree using this date resulted in a median divergence for *CO1* of 1.3%/MY (95% HPD = 0.74% - 2.2%/MY). The same calibration of the *H3* tree resulted in a median divergence for *H3* of 0.064%/MY (95% HPD = 0.043%-0.091%/MY)

There is no significant difference in divergence estimates or node ages using the different calibration strategies (Fig. 2.2), which indicates that our two independent calibrations provide comparable results for dating purposes.

Age comparisons

The age of divergence between South African and Australian Cephalelini found by *CO1* rate calibration is 6.68 MY (95% HPD = 2.4-14.4 MY, Fig. 2.1) and by 16S fossil calibration is 10.46 MY (95% HPD = 4.28-20.24 MY) giving a joint range of possible ages as 2.4 MY - 20.24 MY. This total range is important to note, but it ignores the constraints imposed by our prior knowledge of evolutionary rates of the two gene regions. Rather, the overlapping 95% HPD of possible divergence ages, 5.3MY – 11MY (Fig. 2.3), is most representative of the true age of the divergence, because ages

older than this imply slower rates of mitochondrial evolution than found in more than 30 studies of insect evolution and ages younger than this imply unprecedentedly fast rates of 16S rDNA evolution. Applying this same reasoning to internal nodes in the phylogeny, I find ages of the clades within Cephalelini as follows: *a* = 1.19 MY, *b* = 1.21MY, *c* = 0.99 MY, *d* = 0.1MY, *e* = 0.3MY and *f* = 0.1MY.

Discussion

South African and Australian Cephalelini have diverged very recently relative to the breakup of continents, indicating that the Gondwanan distribution of the tribe is not the result of vicariance and that the group has dispersed between the continents in the last 11MY. In addition, despite the high specificity of Cephalelini species for different Restionaceae hosts, the two groups have not coevolved, since the insects have diversified more recently than their hosts. The youth of Cephalelini also precludes comparison with factors that have broadly affected the evolution of CFR flora and indicates that plants and insects have experienced different evolutionary pressures resulting in diversification, at least in this case.

Evolutionary pattern in the Cephalelini

The lack of monophyly of South African Cephalelini indicates the possibility that the fynbos biome could have been colonised by Cephalelini multiple times, assuming that the direction of dispersal is from Australia to South Africa. This could be indicative of a true polytomy within the group, considering that the morphological phylogeny of Cephalelini by Prendini *et al.*, (1998), which was based mainly on genitalic characters, also found weak support at internal nodes (and weak support overall). If, however, increased gene sampling indicates the South African Cephalelini to be monophyletic, then their current disjunct distribution could be the result of a single dispersal event. Overall, increased taxon sampling of Australian and New Zealand taxa as well as increased gene sampling is necessary to make more precise inferences of the number of dispersal events and the direction of dispersal. Similar to my results, the strongest support in the Prendini *et al.* (1998) phylogeny is for the monophyletic clade containing the three species within *Duospina*. Moreover, these two phylogenies are also congruent in suggesting a close relationship between *C. angustatus*, *C. attenuatus* and *C. uncinatus*. I recover *C. cygnastylus* as a monophyletic species, possibly falling outside of the genus *Cephalelus*. This placement is supported by its divergent stylar morphology relative to *Cephalelus*, which resembles the neck and head of a swan (*cygna* = swan, *stylus* = style). Two of the new species, *Cephalelini spp. nov. 1* and *Cephalelini spp. nov. 2*, have a similar phylogenetic position to *C. cygnastylus*, but with even more divergent genitalic structures, such that

the styles and adeagus of these species more closely resemble that of the Australian outgroup species *Linacephalus foveolatus*.

I find few cases of species-level monophyly within Cephalelini (4 out of 15 species for which multiple accessions were available), but even though both genitalic morphology and gene sequences are insufficiently diverged to provide species-level support using phylogeny estimation, current species delimitations within Cephalelini are still valid from an ecological perspective (specialist host use, Davies, 1988; Augustyn *et al.*, 2013). The lack of phylogenetic resolution at the species levels is probably a function of their recent divergence (< 1.5 MYA), which could easily result in incomplete lineage sorting (Maddison *et al.*, 2006) within species clusters. This possibility is supported by the amount of sequence divergence between Cephalelini species being markedly less than that of *Nesophrosyne* leafhoppers in Hawaii which have radiated in the last 5MY (Bennet & O'Grady, 2012). Bennet & O'Grady (2012) found monophyly of *Nesophrosyne* species and that nuclear and mitochondrial DNA sequences of sister taxa are on average twice as diverged as that of restiohoppers and therefore twice as old. Alternatively it is possible that Cephalelini molecular sequence evolution is much slower than that of *Nesophrosyne*, but I consider this highly unlikely given the range of substitution rates tested here.

Cephalelini and Gondwanan vicariance

The recent divergence between South African and Australian Cephalelini negates the possibility that the observed distribution of Cephalelini is the result of Gondwanan vicariance and therefore implies that intercontinental dispersal has taken place by an unknown method. The evolutionary history of the Restionaceae hosts that Cephalelini leafhoppers utilise (Davies, 1988) also argues against Gondwanan vicariance as an explanation for the distribution of the group (Linder *et al.*, 2003), since the spread of Restionaceae on the African continent is estimated to have started around 66MYA (Linder *et al.*, 2005), which is earlier than the break-up of Gondwana and the separation of Australia from Africa ~180MYA (Wilson *et al.*, 1997). This indicates that the Restionaceae reached Africa by a different route, with Linder *et al.*, (2003) making a convincing case for stepping-stone dispersal of an Australian Restionaceae ancestor across a fragmenting Gondwanaland and into Africa. Divergence dates support dispersal that could have occurred across an Indo-Madagascar landmass connected or close to a not-yet frozen Antarctica. Possible trans-oceanic dispersal in order to cross gaps between separated landmasses is supported by genetic divergence of Restionaceae occurring in Australia and New Zealand that is more recent than the separation of the landmasses (Linder *et al.*, 2003). This theoretical dispersal route for Restionaceae can be tentatively accepted, but the very recent divergence between South African and Australian Cephalelini makes a similar route for their dispersal impossible.

Insects have been known to disperse across very long distances by travelling in jet streams (Gillespie *et al.*, 2013), but this requires a means by which to enter a jet stream, such as flight. Cephalelini are mostly flightless and are therefore not expected to be able to enter jet streams by their own power or to travel the long distances other leafhoppers cover (Taylor & Reling, 1986: seasonal migration of the potato leafhopper across ~250km; Riley *et al.*, 1995: aphids and other insect migrating across India; Anderson *et al.*, 2010: Delphacid migration from Papua New Guinea to Australia; Matsumoto *et al.*, 2013: two leafhopper species migrating across South-East Asia). However, winged female morphs do sometimes occur at low densities (<5%) toward the end of the season (Evans, 1991; Davies, 1988) and the Cephalelini are very well dispersed across the CFR (personal observation; Davies *et al.*, 1988), indicating that rare dispersal events could be a strong factor affecting the distribution of Cephalelini.

Another alternative is that dispersal occurred across mostly-connected landmasses, but that would entail that suitable habitat for leafhoppers existed along the way (Loxdale & Lushai, 1999). Restionaceae are restricted to South Africa and Australia with some representatives in China (Linder *et al.*, 2003), negating the possibility of stepping-stone migration from Australia to southern Africa. A third alternative is dispersal by a winged, generalist ancestor that led to the current species distribution, but this is not a likely or parsimonious scenario considering that Cephalelini on both continents are mostly wingless (Davies, 1988; Evans, 1991). On the other hand, transoceanic rafting may have facilitated the dispersal of Cephalelini, as has been suggested for various other animal taxa (e.g. Leese *et al.*, 2010; Nikula *et al.*, 2013; Townsend *et al.*, 2011; Tolley *et al.*, 2013). While the mechanism of dispersal remains speculative, our study documents one of the only cases of such a recent faunal exchange between South Africa and Australia.

Diversification within the Cephalelini

Although the above results are surprising in that they are contrary to existing ideas about likely evolutionary histories of Cape insect groups (Picker & Samways, 1996), the agreement between the outcomes of the two different calibration strategies provides a high degree of confidence in the dates, considering that information is coming from what are essentially two independent genomes and independent fossil calibration sources. Thus our finding that the Cephalelini in South Africa, a classic fynbos endemic insect group, are recent additions to the fauna suggests that we need to re-examine the assumption that fynbos invertebrates are ancient components of climatically stable environmental refugia within the biome (Picker & Samways, 1996). Further dated phylogenetic analyses need to be carried out for other fynbos endemic insect groups to determine their ages in the CFR and whether the age of Cephalelini is unusually young or not.

Plant diversification in the CFR is thought to have been due in large part to the large variation in topography, soil type and altitude within the region, which effectively 'dissects the landscape' (Linder, 2003), leading to allopatric species distributions. The fact that Cephalelini species have largely overlapping and widespread distributions across the CFR (personal observation; Davies, 1988) indicates that landscape variation may not have played the same role in their evolution than it has in CFR plants. In addition I find Cephalelini diversification to be much more recent than Restionaceae diversification, implying that they have not coevolved in the strict sense (Ehrlich & Raven, 1964; Jermy, 1976), but given the large diversity of Restionaceae species, it is entirely possible that specialisation on different host plant species is leading to the diversification of Cephalelini.

Conclusion

Our cross-validation of the dating exercise using different calibration strategies provides a sound footing from which to estimate divergence time within restiohoppers and between them and their Australian counterparts. The relatively recent diversification within Cephalelini results in poor species-level phylogenetic signal, but the available structure indicates three monophyletic clades of species within Cephalelini. Our findings indicate that molecular and morphological taxonomy run into the same obstacle of low resolution when dealing with recently diverged/currently diverging clades, but the time-stamp which can be gained from molecular analysis provides additional insight into the diversification history of the group.

CFR Cephalelini are very recent additions to the fynbos biome ecosystem, much too recent to have coevolved with their host plants or to conform to any currently described patterns of CFR insect evolution. Their arrival and specialisation in the fynbos indicates the importance of long-distance dispersal events to the accumulation of endemic diversity within the CFR. My findings also suggest a link of hitherto unknown recency between the fauna of the African and Australian continents and specifically between specialist insects in the hyper-diverse fynbos biome and a biome on a different continent, suggesting further investigation of possibly shared fauna between the continents and the effect of this on patterns of diversity.

Table 2.1 Collection localities and gene sampling status for Cephalelini used in this study. Site at which an individual was collected is indicated in brackets after the species name and site coordinates are given in the second and third to last columns. The successful amplification of a gene region for an individual is indicated by a '+' sign, while blank spaces mean no successful amplification. Clade membership as in Fig. 2.1 is indicated in the fifth column. The last column indicates the country of origin of an individual.

	Amplified gene regions				GPS coordinates		
Species (Site)	*CO1*	*H3*	*Sulcia* 16S	Clade	Latitutde	Longitude	Country
C. angustatus (ANYSBERG)1	+	+		b	-33.4997	20.7185	South Africa
C. attenuatus (STELLENBOSCHBERG)	+	+		b	-33.8870	18.9007	South Africa
C. attenuatus (STELLENBOSCHBERG)02	+	+	+	b	-33.8870	18.9007	South Africa
C. attenuatus (STELLENBOSCHBERG)1	+	+		b	-33.8870	18.9007	South Africa
C. attenuatus (VILLIERSDORP)	+	+	+	b	-33.9706	19.1669	South Africa
C. bicoloratus (SEWEWEEKSPOORT)1	+	+		a	-33.3710	21.3416	South Africa
C. bicoloratus (VILLIERSDORP)1	+	+		a	-33.9706	19.1669	South Africa
C. brevipilus (ANYSBERG)	+	+	+	b	-33.4997	20.7185	South Africa
C. brevipilus (SEWEWEEKSPOORT)1		+		b	-33.3710	21.3416	South Africa
C. brevipilus (GAMKABERG)	+	+	+	b	-33.6976	21.9207	South Africa
C. campbelli (CEDERBERG)1	+	+		a	-32.1286	18.8510	South Africa
C. campbelli (GEORGE) 1	+	+		a	-33.9609	22.5376	South Africa

C. campbelli (GEORGE) 2	+	+		a	-33.9609	22.5376	South Africa
C. campbelli (GEORGE) 3	+	+		a	-33.9609	22.5376	South Africa
C. campbelli (SCARBOROUGH)	+	+		a	-33.3873	19.3170	South Africa
C. cygnastylus (LANDDROSKOP)	+			d	-34.0489	19.0099	South Africa
C. cygnastylus (LANDDROSKOP)2	+			d	-34.0489	19.0099	South Africa
C. daviesi (CEDERBERG)	+	+		a	-32.1286	18.8510	South Africa
C. daviesi (STELLENBOSCHBERG)		+		a	-33.8870	18.9007	South Africa
C. ivyae (RONDEBERG)	+	+		a	-33.4167	18.2865	South Africa
C. ivyae (RONDEBERG) 2	+	+		a	-33.4167	18.2865	South Africa
C. linderi (GEORGE)2		+		a	-33.9609	22.5376	South Africa
C. linderi (SEWEWEEKSPOORT)	+	+		a	-33.3710	21.3416	South Africa
C. nivenus (VILLIERSDORP)43	+			a	-33.9706	19.1669	South Africa
C. nivenus (VILLIERSDORP)39			+	a	-33.9706	19.1669	South Africa
C. pickeri (CEDERBERG)01	+	+		a	-32.1286	18.8510	South Africa
C. pickeri (CEDERBERG)2	+	+		a	-32.1286	18.8510	South Africa
Cephalelini spp. nov.2 (PRINGLE BAY)1	+	+	+	e	-34.3258	18.8390	South Africa
Cephalelini spp. nov.2 (PRINGLE BAY)2	+	+		e	-34.3258	18.8390	South Africa
Cephalelini spp. nov.2 (PRINGLE BAY)3	+	+		e	-34.3258	18.8390	South Africa
C. rawsonia (STELLENBOSCHBERG) 1	+	+		a	-33.8870	18.9007	South Africa
Cephalelini spp. nov.1 (TRIPLET) 1		+	+	f	-34.0219	18.9870	South Africa
Cephalelini spp. nov.1 (TRIPLET) 3		+	+	f	-34.0219	18.9870	South Africa
C. spp. nov. (RONDEBERG) 1	+	+		b	-33.4167	18.2865	South Africa
C. spp. nov (RONDEBERG)01	+	+		b	-33.4167	18.2865	South Africa

C. spp. nov (RONDEBERG)1	+	+		b	-33.4167	18.2865	South Africa
C. spp. nov (RONDEBERG)2	+	+	+	b	-33.4167	18.2865	South Africa
C. smithi (SUURBERG)	+	+		a	-33.2854	25.7130	South Africa
C. uncinatus (BRANDSEBAAI)		+	+	b	-31.2716	17.9761	South Africa
C. uncinatus (HONDEKLIPBAAI)	+	+		b	-30.3377	17.3610	South Africa
C. uncinatus (HONDEKLIPBAAI)2	+	+		b	-30.3377	17.3610	South Africa
C. uncinatus (OTTER)1	+	+	+	b	-33.9834	23.6767	South Africa
C. uncinatus (OTTER)2	+	+	+	b	-33.9834	23.6767	South Africa
D. capensis (CEDERBERG)	+			c	-32.1286	18.8510	South Africa
D. capensis (CEDERBERG)1	+			c	-32.1286	18.8510	South Africa
D. capensis (CEDERBERG)2	+			c	-32.1286	18.8510	South Africa
D. sheilae (CEDERBERG)1		+		c	-32.1286	18.8510	South Africa
D. twanella (SWARTBERG) 1	+	+		c	-33.3423	22.0411	South Africa
D. twanella (SWARTBERG)3	+	+	+	c	-33.3423	22.0411	South Africa
L. foveolatus (AUSTRALIA) 1	+	+		-	-20.8	115.4	Australia
L. foveolatus (AUSTRALIA) 2	+	+		-	-20.8	115.4	Australia
L. foveolatus (AUSTRALIA) 3	+	+		-	-20.8	115.4	Australia
L. foveolatus (AUSTRALIA) 4	+	+		-	-20.8	115.4	Australia

Table 2.2 Details of the primer pairs and the annealing temperatures used in this study. LCO-HCO is an universal insect primer pair used for barcoding purposes. H3F-H3R is commonly used to investigate evolution of insect nuclear genes. 10FF-1370R is a *Sulcia* specific primer pair used to isolate symbiont amplicons from whole-body insect extractions.

Primer name	*Primer sequence*	*Annealing temperature*	*Reference*
LCO1490	GGTCAACAAATCATAA AGATATTGG	55 °C	Folmer *et al.* 1994
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
H3F	ATGGCTCGTACCAAGCAGACGGC	57-42 °C (touchdown)	Ogden *et al.* 2003
H3R	ATATCCTTGGGCATGATGGTGAC		
10FF	AGTTTGATCATGGCTCAGGATAA	57.8 °C	Takiya *et al.* 2006
1370R	CGTATTCACCGGATCATGGC		

Table 2.3 Genbank accesion numbers (sequences from Takiya *et al.*, 2006) for the three outgroup Cicadellid species used to calibrate a common root node for Cephalelini in order to compare rate and root age estimates between different calibrations.

Outgroup species	Gene region		
	CO1	*H3*	*Sulcia* 16S
Pagaronia tredecimpunctata	AY869732.1	AY869755.1	AY676911.1
Paraulacizes irrorata	AY869737.1	AY869762.1	AY676912.1
Proconosama alalia	AY869742.1	AY869768.1	AY676906.1

Table 2.4 Summary information of the genetic analysis of the three gene regions used in this study. Model choice is reported as those models that received the best Decision Theoretic (DT) score. Clock models were determined in Mega v. 5.1 and verified in BEAST.

Primer name	Gene (Genome)	Aligned amplicon length (bp)	Model (DT)	Clock model	Mean rate
LCO1490-HCO2198	*CO1* (insect mitochondrial)	351	TIM1 + G	Relaxed, uncorrelated exponential clock	1-2.3 % per MY (from Papadopoulou *et al.*, 2010)
H3F-H3R	*H3* (insect nuclear)	268	K80 + G	Relaxed, uncorrelated exponential clock	0.09% per MY*
10FF-1370R	16S (symbiont rDNA)	1037	HKY	Strict clock	0.015% per MY*

*estimated from *CO1* root node calibration

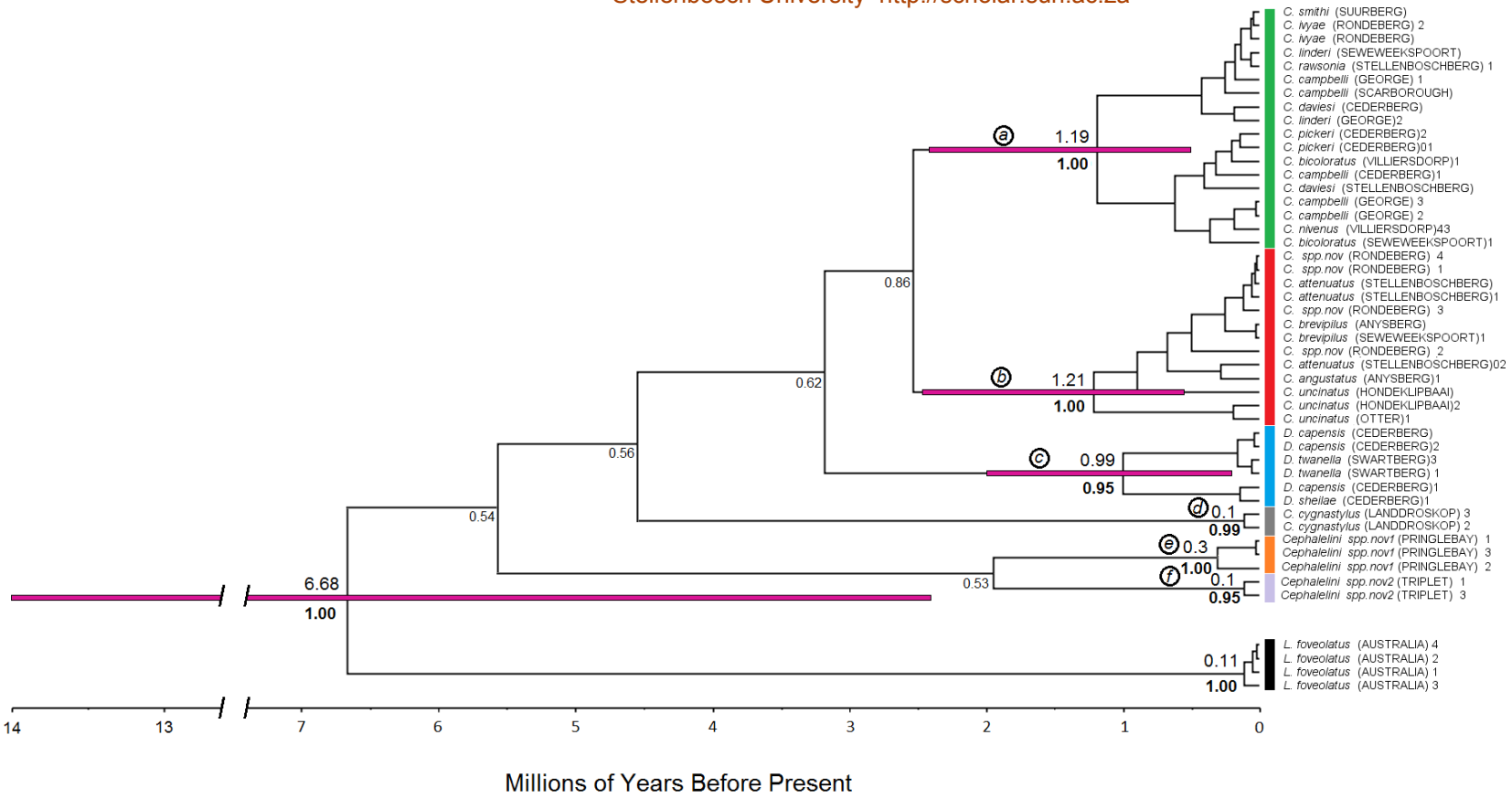

Figure 2.1 Bayesian chronogram of concatenated analysis of Cephalelini *CO1* and *H3*. Node ages in millions of years before the present are indicated above branches leading to that node and posterior probabilities are indicated below the branches. Significant support values for nodes are indicated in bold (posterior probability > 0.91; Zander, 2004). Divergence times were estimated using a ranged rate calibration of 1 - 2.3% divergence / MY (Papadopoulou *et al.*, 2010) and an uncorrelated exponential clock. The age of divergence between South African and Australian Cephalelini is indicated at the root (median of 6.68 MY, 95% HPD = 2.4-14.4 MY). Monophyletic species clusters within the South African Cephalelini are indicated by encircled letters above branches leading toward each monophyletic clade. Coloured bars at the tips of the tree also indicate the different clades. *a*, green, and *b*, blue, are two monophyletic species clusters within the genus *Cephalelus. b* contains the newly-discovered species *C. spp. nov.* and *c* is a clade composed of all *Duospina* species. *d*, gray, contains one species from the genus *Cephalelus. e*, orange, and *f*, lilac, each contain one of the newly discovered species. The black bar indicates the Australian outrgoup. Shaded bars at nodes indicate the 95% HPD of node heights in the tree. Slashes in the root node error bar and the timeline indicate an abbreviation of the timeline.

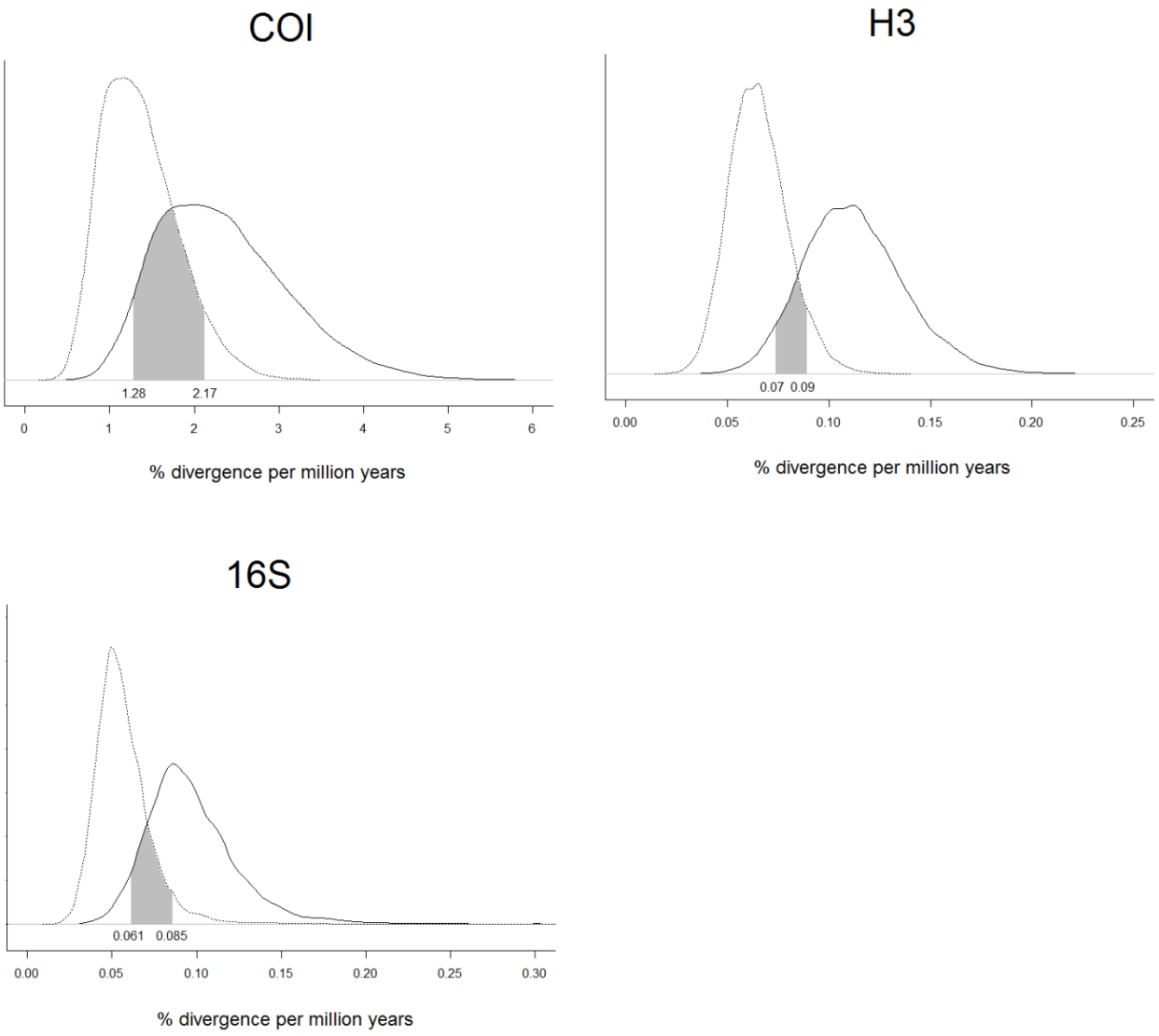

Figure 2.2 Overlaid density plots of BEAST parameter estimates comparing differences in divergence rates of the three different gene regions (left column = *CO1*, middle column = *H3*, right column = *Sulcia* 16S) using different calibration strategies (solid lines = *CO1* rate calibration from Papadopoulou *et al.*, 2010; dotted lines = *Sulcia* 16S fossil calibration from Moran *et al.*, 2005). Shaded areas under curves indicate the overlapping range of 95% highest posterior densities of parameter estimates, with numbers under these areas indicating the minima and maxima of these overlapping areas.

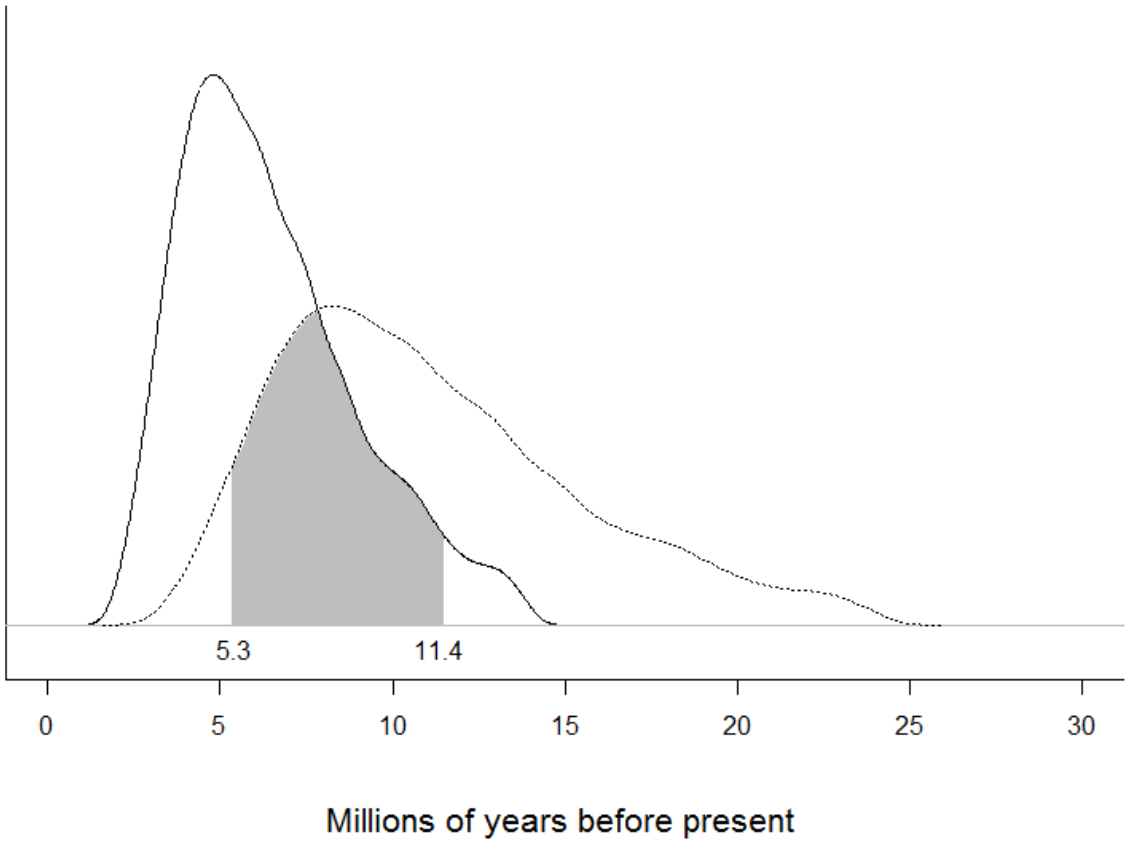

Figure 2.3 Overlaid density plots of the BEAST divergence age estimates between South African and Australian Cephalelini using *CO1* rate calibration (solid line) and *Sulcia* 16S fossil calibration (dotted line). The shaded area under the curves indicate the overlapping 95% highest posterior density for the estimates of divergence age and numbers below these indicate the youngest and oldest likely ages given both calibration strategies.

Chapter 3

Dissecting the evolution of host range and preference of Cephalelini leafhoppers on fynbos Restionaceae: a phylogenetic approach

Abstract: Herbivorous insects evolve to become more specialised in their host associations, at the cost of fitness on a larger range of hosts. Investigating the evolution of host use and specialisation makes it possible to determine the factors responsible for increased specialisation as well as the evolutionary effects of specialisation. Here I used evolutionary relationships of South African Cephalelini leafhoppers and their Restionaceae hosts to investigate the evolution of host preference and host range as well as the evolutionary relationships between used and avoided host plants. I used Pagels' λ, D, a parsimony null model test and Blomberg's K to test for phylogenetic conservatism of several traits relating to host use and specialisation. I found no evidence of evolutionary constraint on Cephalelini host preference evolution at the clade or genus levels in the Restionaceae. In contrast, preference for Restionaceae tribes exhibits strong phylogenetic signal, with the major clades of Cephalelini exhibiting preferences for different tribes of Restionaceae. Broader analyses of the full range of hosts used by Cephalelini taxa confirmed that host range of the main Cephalelini clades is also restricted to the two tribes of Restionaceae. I conclude that Cephalelini specialisation has resulted in their evolution tracking that of their Restionaceae hosts. Despite variation in degree of specialisation between species, there is no trend toward increased specialisation within Cephalelini.

Introduction

The evolution of host plant specialisation by herbivorous insects has interested evolutionary biologists for decades (Benson *et al.*, 1975; Jermy, 1984; Bernays & Graham, 1988) due to the often highly specific nature of these associations (Mitchell, 1981; Ehrlich & Murphy, 1988) and the high diversity of herbivores relative to non-herbivorous groups (Mitter *et al.*, 1988). When considering the number of hosts that an insect species uses as the width of its niche, then reduction in niche width can be expected to occur over time in response to factors such as increasing interspecific competition, increase in preferred host abundance or decrease in the availability of enemy-free space (Van Valen, 1965; Ackerman & Doebeli, 2004; Roughgarden, 1972), or simply as a function of adaptations that increase fitness on one host that come at the cost of fitness on a broader range of hosts (Futuyma & Moreno, 1988; Via, 1984; Robinson *et al.*, 1996). Such a decrease in niche width is equal to an increase in specialisation and easily measurable as the number of available hosts used by an insect species. The cost of specialisation can also be investigated by determining the host range of an insect species, which is simply the total number of hosts on which an insect occurs, whereas a preferred host is at the top of a choice or fitness hierarchy of host use (Futuyma & Moreno, 1988). The expectation is that host range will decrease as the efficiency of specialisation increases.

Ehrlich & Raven (1964) outlined the hypothesis that specialist interactions, mainly chemical defense by plants and insects' attempts to breach such defenses, result in reciprocal evolutionary pressures over time which reinforce specialisation and lead to coevolution. Within the coevolutionary framework, a less reciprocal description of the hypothesis was put forward by Jermy (1976), which describes the interaction as asymmetrical, where the evolution of insects is influenced by the evolutionary history of their hosts to a much greater degree than vice versa. This was done in light of the fact that plants are often colonised by a variety of herbivore species and generally have much longer generation times than insects, causing coevolutionary adaptation from their side to be diffuse (Janzen, 1980) across several insect species, rather than specific to one (but see for example Hougen-Eitzman *et al.*, 1993). Jermy (1976) termed this non-reciprocal process 'sequential evolution' to separate it from the idea of coevolution but to still emphasize that the insects are following some constraints imposed by the evolution of their hosts, in contrast with simply evolving onto random hosts. Subsequent to Jermy's (1976) postulation, research suggests that sequential evolution of insects onto their plant hosts is the rule rather than the exception (Kergoat *et al.*, 2011).

Sequential evolution and coevolution result in similar phylogenetic signals, namely that the phylogenetic trees of the interacting groups will have similar branching patterns. The difference is that there will be a temporal disjunction in the case of sequential evolution (Moran 1999; Farell

2001), whereby the sequentially evolving group (usually the insects) is tracking the evolution of the group with which they interact (usually the plant hosts) (as seen with Psyllids and legumes: Percy *et al.*, 2004). Evolutionary tracking takes place due to the assumed phylogenetic similarity of plant defense chemicals that change over time and the likewise phylogenetic similarity in insect adaptations in overcoming these plant defenses (Ehrlich & Raven, 1964; Ehrlich & Murphy, 1988). Therefore, the evolution of a group of specialist insects can be expected to bear a strong imprint of the evolution of the plants on which they specialise, even if they are not coevolving.

The strength of these evolutionary imprints can be determined by the analysis of phylogenies of interacting groups of plants and insects along with traits relevant to the interaction (Pagel 1997), such as host preference and host range of insects. One of the first methods for testing the effect of phylogeny on trait evolution uses parsimony to test the effect of speciation (branching events in a phylogeny) on trait evolution by comparing observed trait changes against a null model (Maddison & Slatkin, 1991). As molecular phylogenies became more prevalent and branch lengths became accurately estimable, models of trait evolution arose taking branch lengths into account, rather than just branching events (speciation) (Pagel 1997; Pagel 1999; Fritz & Purvis, 2010). Models like Pagels' λ (Pagel, 1999) and D (Fritz & Purvis, 2010) can only be used with discrete traits, whereas the more recently developed metrics, e.g. Blomberg's K (Blomberg *et al.*, 2003), allows for strength and significance testing of the evolution of continuous characters.

The South African insect tribe, Cephalelini (Hemiptera: Cicadellidae) (hereafter restiohoppers) occur exclusively (Davies 1988) on Restionaceae (hereafter restios) host plants (Augustyn *et al.*, 2013) in the fynbos biome. Previously, our rate-calibrated molecular phylogenetic hypothesis for the evolution of restiohoppers in conjunction with a dated phylogeny of the restio host plants (Linder *et al.*, 2005) indicated that the major diversification of restios occurred some 20 MY before the diversification of restiohoppers. This completely negates the possibility of coevolution in the strict sense between the two groups, because reciprocity of selective pressure is impossible if divergence within the two groups were not contemporaneous (Moran *et al.*, 1999; Farrell 2001; Percy *et al.*, 2004). However, given the specialisation of restiohoppers on their restio hosts, the likelihood that they are tracking the evolution of their hosts remains open to investigation.

We ask the following questions in order to investigate the evolution of restiohopper host use in light of their own evolution and that of their restio hosts:

1) Is restiohopper herbivory restricted to specific clades within Restionaceae?
2) a) Do closely-related restiohoppers prefer the same restio hosts?
   b) Do restiohoppers prefer closely related restio hosts?
3) Lastly, does a restiohoppers species' host range consist of closely-related restios?

4) Finally, is there an evolutionary trend towards increased specialisation within restiohoppers?

Methods

Sampling and host associations

Sampling was carried out at 52 sites across the South African fynbos to cover the known distributional range of restiohoppers as described in Davies (1988). Collection of insects was done by vacuum-suctioning of all restio species present at a site using a leaf blower modified by addition of a fine mesh bag to the front of the intake tube. Insects were removed from the mesh bag and stored in 96% ethanol for later identification. Forty individual plants of each restio species present at a site were sampled (20 male and 20 female plants where sexes were easily discernable) in order to obtain a standardised measure of restiohopper relative density between plant species and across sites. Voucher specimens of the vegetative and floral parts of each plant species were collected and pressed for later identification with Delta-Intkey identification software (Dallwitz, 1980) and the Restionaceae dataset (Linder, 2011). Restiohopper specimens were identified to the species level by dissection of the genitalia and using species descriptions from Davies (1988) & Prendini (1997).

Restiohopper species were matched to the host species on which they were collected to determine host associations. Insect densities on each host species, which were used to assign host preference and host range, were determined as the number of insect individuals per 40 host plant individuals. In instances where a restio species was sampled at multiple sites, insect density per host was averaged across sites. Very rare host associations, represented by a single restiohopper individual, were removed from the analyses to reduce the potentially confounding effects of accidental by-catch.

Phylogenies

The insect phylogeny used here is the result of Bayesian phylogenetic analysis of multiple accessions per species of two genes (*H3* and *CO*I) from a previous study of 17 restiohopper species (see Chapter 2). I used a single tree (highest posterior clade probability) for the analyses, rather than averaging across a large set of trees, because the clades I use to test my hypotheses are all well-supported, with posterior probabilities of clades ranging from 0.95-1.00 (Zander, 2004). I randomly pruned the selected tree to contain a single accession per species, beacuse analyses of trait evolution do not allow for multiple accessions per species.

The restio host-plant phylogeny is based on the analysis of multiple plastid regions from 345 species combined into one analysis in BEAST. The program was run for 100 000 000 generations, sampling every 2000th generation. Two fossil calibrations were used; one for the age of African Restionaceae (normal prior, mean = 66 MY, standard deviation = 2.0) and one for the age of the limestone-specialist clade African Restionaceae (normal prior, mean = 3 MY, standard deviation = 0.5) (Linder *et al.*, unpublished data).

Test of phylogenetic signal

I used four different techniques to test for phylogenetic signal to explore the evolution of host use of restiohoppers, namely: Pagels' λ (Pagel, 1999), D (Fritz & Purvis, 2010), a null-model test of minimum character state change using parsimony (Maddison & Slatkin, 1991) and Blomberg's K (Blomberg *et al.*, 2003), a method of testing phylogenetic signal of the evolution of continuous traits. Each approach has advantages particular to some aspect of the datasets I was working with: Pagels' λ is useful because analysis of traits allow for more than two character states, D is useful because it is a scaled metric that can be compared between datasets and provides further information on the degree of observed phylogenetic signal, Blomberg's K is useful because it can test the evolution of continuous characters and minimum character change testing is useful because it is less sensitive to small sample size than the other three metrics and provides a measure of the effect of speciation on trait distributions among species rather than the effects of branch lengths.

Pagels' λ (Pagel, 1999) - Tests of Pagels' λ provide a measure of the degree of branch length transformation that best explains the distribution of trait states at the tips of a phylogeny. λ = 1 leaves branch lengths as is and λ = 0 makes all branch lengths equal, removing the effect of topology on the covariance of traits. Maximum likelihood analysis is used to find the most likely λ value (between 0 and 1) given the observed distribution of character states on a phylogeny. The likelihood of the observed model is compared to the likelihood of a model with λ = 0 using a likelihood ratio test, in order to determine whether the trait exhibits significant phylogenetic signal. The higher the value of λ, the greater the phylogenetic signal of the trait being analysed.

D (Fritz & Purvis, 2010) - D is a test statistic calculated by summing sister clade differences of binary characters on a phylogenetic tree and scaling these against models of random evolution of the binary trait and Brownian motion (BM) evolution of the binary trait respectively. Random evolution of traits is simulated by randomly reassigning trait states across the tips of the tree and then summing sister clade differences. Simulation of BM evolution of the binary trait is achieved by allowing an arbitrary continuous trait to evolve along the tree by random walk with constant trait variance over time (Felsenstein, 1985) and using a threshold model to convert continuous traits to one of the binary states while also constraining the prevalence of each binary state to be equal to the observed prevalence. The random and BM simulations of trait evolution are then

randomly permuted a large number of times to generate distributions for each model of trait evolution against which the observed sum of sister clade differences can be statistically compared. D is calculated as follows

$$D = [\sum d_{obs} - \text{mean}(\sum d_b)]/[\text{mean}(\sum d_r) - \text{mean}(\sum d_b)]$$

where d_{obs} is the observed sister clade differences, d_b is the BM modeled sister clade differences and d_r is the randomly modeled sister clade differences. Therefore D is a ratio, with D = 1 describing random evolution of a binary trait and D = 0 describing BM evolution of a binary trait. D is useful for two reasons. First, it provides a metric of phylogenetic signal that is comparable between datasets. Second, values of D less than 0 and greater than 1 can provide additional information on trait distribution with regards to phylogeny, indicating strongly conserved and strongly overdispersed traits respectively. The lower the value of D, the greater the phylogenetic signal being analysed.

Minimum character-state change (Maddison & Slatkin, 1991) - With this approach, the minimum number of character state changes along the phylogenetic tree given the observed distribution of tip states is calculated using parsimony methods (Farris, 1970; Fitch, 1971). The tip states are then randomly reshuffled a large number of times and the minimum number of character state changes recalculated every time to generate a null distribution of the expected number of state changes along the tree. The observed number of state changes are then compared against the null distribution to test whether more or less changes than expected have taken place. This test is similar in effect to the speciational model of trait evolution described in Mooers *et al.* (1999) in that branching events rather than branch lengths are measured for their effect on trait evolution.

Blomberg's K (Blomberg *et al.*, 2003) – K is a metric used to compare the strength of phylogenetic signal of continuous traits on a phylogeny. First, significant departure from random trait evolution is tested by computing the mean squared error (MSE) of the observed traits on the phylogeny using phylogenetic generalised least squares (PGLS) (Garland & Ives, 2000). Tip states are then randomly reshuffled, MSE is again calculated using PGLS and this is repeated a large number of times, randomising tip states each time. The values of the randomised MSE are used to generate a distribution against which the MSE of the observed data can be compared to determine if more or less signal than randomly expected exists in the data. Second, K is calculated to determine observed signal strength relative to signal strength expected under Brownian motion (BM). This is accomplished by first determining the ratio of the observed MSE of the data over the expected MSE. The expected MSE is the MSE relative to the phylogenetically corrected mean of the trait being tested. Secondly, this same ratio (observed MSE over expected MSE) is calculated for a modification of the data constraining it to follow a pattern of BM evolution. K is then the ratio of the first ratio over the second ratio. This means that values of K greater than 1 implies more

phylogenetic signal than expected under BM evolution and K less than 1 implies less phylogenetic signal than expected under BM evolution.

All analyses in this study were conducted in the R Statistical Environment v.3.0.2 (R Core Team). Pagels' λ was calculated using the fitDiscrete function in geiger v.3.1 (Harmon *et al.*, 2008) with a wrapper function written for this study (available upon request) used to calculate the significance of the likelihood ratio of the model with $\lambda = 0$ and a model with the maximum likelihood estimate of λ given the observed trait values. Calculation of D and significance testing of D against the random and BM models of trait evolution was performed using the phylo.d function in caper v.0.5.2 (Orme *et al.*, 2013). Null modelling of minimal speciational trait change using parsimony according to Maddison & Slatkins' (1991) "Fixed Tree, Character Randomly Reshuffled" model was carried out using a function written by Enrico Rezende (https://stat.ethz.ch/pipermail/r-sig-phylo/2011-March/001035.html).

Host preference

Host preference was assigned to insect species in order to separate hosts on which insects potentially have high fitness from sub-optimal hosts. I did this by using insect densities as a measure of relative fitness on a host. Host preference was determined by ranking the densities of a restiohopper species across restio host species (average number of individuals per 40 restio plants) from highest to lowest, and then determining how much each successive host contributes to the overall abundance of that insect species. I chose 50% as an arbitrary cut-off to assign preferred hosts, meaning that the ranked hosts contributing the first 50% of the total abundance of an insect species are considered to be the preferred hosts.

We used the restiohopper phylogeny to test insect evolutionary preference for taxonomic/phylogenetic levels of preferred restio hosts. I tested phylogenetic signal at the tribal (Briggs *et al.*, 2009), clade (from restio phylogeny, Table 3.1) and genus (Linder & Hardy, 2010) levels, in order to 1) determine whether closely-related restiohoppers use closely-related restio hosts and, if so, to 2) determine at what host taxonomic level the phylogenetic signal of host-use breaks down. This was accomplished by assigning the different taxonomic ranks of hosts preferred by each restiohopper species as character states on the restiohopper phylogeny and testing for phylogenetic signal using Pagels' λ, *D* (where characters were binary) and the parsimony null model test.

We use the restio phylogeny to provide an additional perspective on the evolution of restiohopper herbivory patterns and host range. I first tested for possible phylogenetic sampling bias of restio species that could affect subsequent analyses. I did this by assigning being sampled or not as a character state to each of the restio species in the tree and testing for phylogenetic signal using

Pagels' λ, *D* and the parsimony null model test. I also determined whether host use by Cephalelini as a group is restricted to certain clades within Restionaceae by testing phylogenetic signal of all hosts utilised and avoided by restiohoppers on a restio sub-tree consisting only of the sampled hosts using Pagels' λ and the parsimony null model test. Thereafter I examined the evolution of preferred hosts of different restiohopper clades to compare with the results from analysis of the restiohopper phylogeny. I determined whether closely-related restiohoppers prefer closely-related hosts by using well-supported clades of insects and assigning the clade to which an insect belongs as the trait state to the host which it prefers and testing phylogenetic signal of the clades on a sub-tree of the restio phylogeny consisting of all the preferred hosts of the different restiohopper species using Pagels' λ and the parsimony null model test.

Host range and specialisation

In addition to analyses of host preference I furthered explored host-use evolution in restiohoppers by testing whether the range of hosts utilized by each restiohopper species is phylogenetically constrained. The host range of a restiohopper species was defined as all restio species which supported a density of more than one insect per 40 individuals of the host species. In order to determine if the evolutionary relationship between host plants constrains the range of hosts a single restiohopper species can occupy, I assigned host-use (1 = used, 0 = unused) as trait states on a sub-tree of the restio phylogeny and tested for phylogenetic signal of each individual species' host range using Pagels' λ. For each host range test a subtree of the restios was used that consisted only of the host species encountered by a single restiohopper species (i.e. all restio species occurring at all sites at which a restiohopper occurred).

We explored host range in more detail by testing whether phylogenetic similarity between hosts within a species' host range is indicative of host quality, with insect density on a host used as a proxy for host quality. I assigned densities of an insect species on hosts in its range as a continuous character trait and tested signal using Blomberg's K.

Lastly, I investigated whether the host ranges of restiohopper clades are restricted to restio tribes by comparing the aggregate host range of insect clades between host tribes. This was accomplished by first finding the proportion of encountered hosts that a species used within a tribe of hosts and aggregating this for all species within a clade. These aggregate proportions were then compared between host tribes for different insect clades using z-tests.

Finally, I define an individual insect species' relative host range as the proportion of hosts used versus hosts encountered in order to obtain a relative measure of specialisation for each species. This allows me to investigate the evolutionary trajectory of specialisation within Cephalelini by

assigning the relative host range of each restiohopper species as a continuous character to tips on the restiohopper phylogeny and testing for evolutionary signal using Blomberg's K.

To visualise the likely ancestral states of traits, I performed reconstruction of ancestral character states for discrete traits with significant phylogenetic signal using the ace function in ape v.3.0-11 (Paradis *et al.*, 2004).

Results

Sampling and host associations

Sampling over three years and all seasons across the known range of South African Cephalelini, recovered 1476 individuals of 17 restiohopper species at 52 different sites. I sampled 101 of 345 known restio species and found restiohoppers on 39 of these species (see Fig. 3.1). Some hosts were occupied by multiple restiohopper species, bringing the total number of recorded host associations to 48. An additional seven associations involving a single restiohopper individual were recorded, but these were disregarded in host-use analyses.

Phylogenies

The restiohopper phylogeny used in our analyses has three well supported (posterior probability > 0.91 clades of species (Fig. 3.2A). Clade D contains the three known species of the genus *Duospina*, clade C1 contains 10 species of the genus *Cephalelus* and clade C2 contains 5 species of the genus *Cephalelus* (Fig. 3.2). Although there is further structure within clade C1, when examining the original tree consisting of multiple accessions per species it is seen that this structure renders some species paraphyletic. Therefore I ignored this extra level of structure, since it would not be compatible with species-level host assignments. The branching order of the three clades is not well-resolved (posterior probability = 0.73), which makes meaningful interpretation of maximum likelihood character state estimation at the root of the phylogeny impossible.

The Wildenowieae and Restioneae tribes form two well-supported, monophyletic clades within the restio phylogeny (Fig. 3.2A). There are also various well-supported clades within each tribe (Fig. 3.1), some of which contradict current generic limitations, such as that of *Restio*, *Willdenowia* and *Hypodiscus*.

Host preference evolution

Based on our host preference criteria, 13 of the 17 restiohopper species were assigned a single preferred host, four species had two preferred hosts and a single species had three preferred hosts

(Table 3.1 and Fig. 3.3). Multiple preferred hosts per species were incorporated into the analyses by running multiple analyses with each possible host preference for a species and considering all results. Significance or lack thereof was not different for any of the different combinations.

We found no significant tendency for closely-related restiohoppers to prefer the same genus or clade of host, but they do prefer hosts of the same tribe. D and λ are significant (and highly similar taking both of the host tribe preferences of *C. shortnose* into account), but the parsimony null-model test is not significant (Table 3.2 and Fig. 3.2 D) at the restio tribe level. The inconsistency of these results is best explained by the effect of branch lengths on analysis, since both D and λ take branch lengths into account, but the parsimony null model test does not.

Testing phylogenetic signal on the restio phylogeny I found no phylogenetic bias in our sampling of restio hosts (Table 3.2), as indicated by a λ of 0 and D not different from 1 and different from 0. The parsimony null model test, however, indicated significant phylogenetic signal. This could be problematic for downstream analyses using a pruned tree consisting of only the sampled restios, but since the parsimony null model test does not take actual branch lengths into account, while λ and D do, I felt it was justifiable to disregard any subsequent effects other than on the parsimony null model test.

There was no statistical indication of closely-related hosts being used or avoided, as indicated by λ, D and the parsimony null model test (Table 3.2). I found significant phylogenetic similarity between hosts preferred by the three clades of restiohoppers (Fig. 3.2 D), as indicated by λ and the parsimony null model test. The distribution of preferred hosts of the three clades also supported the tentative conclusion reached by analysis of the Cephalelini phylogeny, namely that host use was partitioned between restio tribes.

Evolution of host range and specialisation

Host ranges spanned one to 11 host species per insect species and overlapped somewhat between insect species. Relative host range was also fairly variable, ranging from 6.3% to 33 % of encountered hosts being utilised by an insect species.

We found that the host ranges of *C. angustatus* ($\lambda = 0.62$, $p<0.005$) and *C. rawsonia* ($\lambda = 0.72$, $p<0.005$) consisted mainly of closely-related hosts, but that the other 11 restiohopper species host ranges reflected a random phylogenetic sample of the available hosts. *C. brevipilus, C. daviesi, C, ivyae* and *C. shortnose* were excluded from the host range analyses, since they only exploit a single host, which makes analysis meaningless (Münkemuller *et al.*, 2012). Testing whether densities on a host within a species host range support this pattern, I found that the pattern holds for *C. angustatus* ($K = 0.1$, $p<0.005$), but not for any of the other species.

Blomberg's K indicated that closely-related restiohoppers do not ($K = 0.038$, $p = 0.8$) have similar relative host ranges (our measure of specialisation), implying that there is no overall trend in Cephalelini evolution towards increased specialisation.

By pairwise comparison using a z-test, I found that the proportion of encountered hosts used by each insect clade is statistically similar to one another, with clades using roughly a sixth of the hosts that they encounter (proportions of encountered hosts used: Clade D = 0.14, Clade C1 = 0.12, Clade C2 = 0.16). However, within each clade I find differences for encountered hosts used per tribe (Fig. 3.1), with Clades D and C1 using significantly more of the encountered Restioneae tribe hosts and Clade C2 using significantly more of the Wildenowieae tribe species, as indicated by a z-test ($z = -3.34$, $p < 0.0005$).

Discussion

Restiohopper host use has tracked the evolution of their hosts, with closely-related hoppers preferring either Restioneae or Wildenowieae tribes of Restionaceae. Restiohoppers occur on roughly only a third of available restio hosts that they encounter and show preference for even less, mostly one or two hosts per species. Restiohopper host range is also conserved at the level of host tribe, but there is no tendency for hoppers to utilise or avoid clades of restio species. Even though species vary in their degree of specialisation, there is no trend towards increased host specialisation within restiohoppers.

Restio evolution and host use

The lack of phylogenetic signal in the distribution of hosts used and avoided by restiohoppers warrants attention, especially considering that classical coevolutionary theory (Ehrlich & Raven, 1964), the non-reciprocal revision of the theory (Jermy 1974) and many empirical studies of the chemical similarity of defense compounds of related plants (reviewed in Wink, 2003), predict that closely related plants will harbour or deter closely related insect herbivores. Considering that closely-related restiohoppers prefer the same tribe (but not clade or genus) of restio hosts and that chemical defenses within these tribes can be expected to be similar, any uninhabited host species within these tribes represents an unoccupied potential niche (Hutchinson 1957; Futuyma *et al.*, 1988). Although it is possible that this is a sampling artifact, I conclude otherwise, since sampling has spanned several seasons over three years and analysis indicated that there is no phylogenetic signal of sampling bias, nor does it seem possible that a large number of species could have been overlooked. One explanation is that the absence of restiohoppers is the result of a restio trait (or traits) that is not under any phylogenetic constraint or a trait (or traits) that has arisen

independently in multiple restio taxa (i.e. by convergent evolution). Alternately, restiohoppers might be excluded from hosts through interspecific interactions such as competitive exclusion by other herbivores or as a result of increased susceptibility to predation. Overall, the lack of phylogenetic similarity of utilised and avoided hosts could be the result of adaptive host choice being less a species property and more a population property (Fox & Morrow, 1981), which would cause host choices to vary between populations based on local conditions rather than overarching phylogenetic constraints.

Host preference evolution

Analysis of restiohopper evolution and contemporary host use patterns indicated that a preference for either the Wildenowieae or Restioneae tribe of hosts (Briggs & Linder, 2009; Fig. 3.2A) is a shared trait within certain clades within Cephalelini (Fig. 3.2B). The analytical methods used to detect this pattern gave mixed results which is probably the result of the low number of species used (18 vs a suggested minimum of 25 by Münkemuller *et al.*, 2012). However, our analysis of the larger restio phylogeny in conjunction with restiohopper preference patterns support tribal affinity among closely-related restiohoppers and further indicated the phylogenetic similarity of hosts within restiohopper clades (Fig. 3.2A), indicating that restiohopper host use has tracked the evolution of their hosts. It is interesting that restiohopper host preference is not phylogenetically conserved at a finer taxonomic scale than host tribe, since individual species of restiohopper tend to be highly specialised in their host preference. It could be that similar factors to those affecting the patterns of utilisation and avoidance are in effect here.

Host range evolution

Restiohopper host range was partitioned at the same host tribal level as host preference, as indicated by comparison of relative host range of the different clades on the different tribes, but the signal was not detectable using the standard tests of phylogenetic signal. This seems to result from the observed pattern of species host ranges, where a species will often encounter host sister taxa and only occur on one of the two species, resulting in a checkered distribution of used hosts along the tips of the restio phylogeny (Fig. 3.1). The documented diversity of within-genus defensive chemicals (e.g. flavonol glycosides within the obsolete genus *Chondropetalum*; Harborne *et al.*, 1984) means this checkered distribution of host choice could result from sister taxa that have diverged sufficiently in their defensive chemistry to differentially exclude restiohopper species. Considering the age of restio genera (last diversification between genera 12-14 MYA; Linder *et al.*, 2005) and the suggested lability of the evolution of plant chemical defenses (Harborne, 2000), it could be that restio chemical defenses have been randomly deviating and converging, which would result in the checkered matching of restiohopper host use to restio phylogeny.

The similar evolutionary pattern of host preference and host range being conserved at the level of host tribe indicates that even though different clades of insects have labile host ranges within the tribes which they prefer; their associations seldom stray outside these tribes. Considering the age of the tribes (43-55 MY; Linder *et al.*, 2005), this could indicate the conservation of a broad suite of chemical defenses within these tribes that diverged at the point where the tribes diverged, which now differentially excludes restiohoppers. However, considering the specialised sheath-like morphology of the insects speculated to be involved in cryptic avoidance of predators (Davies, 1988), it is possible that chemistry is not the only or even the main factor influencing insect host choice and that morphological matching of hosts also plays a role.

Another possible explanation is that the evolutionary tracking of restios by restiohoppers is 'fuzzy' beyond the level of host tribe due to the fact that the two groups are not coevolving in the true sense (see Chapter 2). There is no reason why the evolution of restiohoppers should exactly follow the evolution of restios, especially when considering the old age of restios (22-66 MY) versus the young age of restiohoppers (< 6MY). It could be that secondary metabolite detoxification strategies of restiohoppers are evolving on completely different trajectories than restio chemical defenses have evolved, which would result in closely related insects potentially specialising on divergent hosts, leading to the breakdown of phylogenetic signal of host specialisation.

Specialisation

The fact that I detected no trend towards increased specialisation within restiohoppers is counter to some expectations of the evolutionary trajectory of specialisation (Fry, 1996; Futuyma & Moreno, 1988; Nosil, 2002), but could be explained by the relatively young age of the tribe Cephalelini (5-11 MY, Chapter 1) when compared to a leafhopper group such as the genus *Nesophrosyne* in Hawaii (Bennet & O'Grady, 2013). Considering the recency of restiohopper-restio interactions, it is possible that variation in host choice between populations (Fox & Morrow, 1981) or even between individuals (Bolnick *et al.*, 2003) successfully obscures phylogenetic signal of trends in specialisation at the species level. This variation could decrease over time as selective sweeps across the distribution of a species causes host choice to become fixed in the evolution of a species. It is also possible that the phylogenetic resolution (within insect tribe, between three clades within two genera) at which I investigated the evolution of specialisation is too fine to detect trends and that the restiohoppers as a whole represent a group that has become increasingly specialised relative to other leafhopper tribes.

Conclusion

Restiohoppers are highly specialised in their use of restios. Their host preference and host range have differentiated along the same phylogenetic boundaries within their restio hosts, namely that

of the Restioneae and Wildenowieae tribes. However, the checkered distribution of host range within tribes indicates the presence of an unknown trait that is not constrained by phylogeny that is acting to exclude restiohoppers. The relatively recent diversification of restiohoppers supports the idea that population-level differences in host preference are important to the outcome of the evolution of specialisation within species, which suggests that conservation of these species' varying host ranges across their distributions are important to maintain ongoing evolutionary processes important to diversification in the group.

Table 3.1 Cephalelini clades, preferred host species, host clades (see Methods section), host genera and host range used to test for phylogenetic signal.

Cephalelini species	**Ceph clade**	**Preferred host species**	**Preferred tribe**	**Preferred clade**	**Other hosts**
C. angustatus	C2	*Wildenowia incurvata, Mastersiella spathulatha, Hypodiscus arisatatus*	W	Hypo-Wil	*Cannomois primosii, Cannomois robusta, Cannomois scirpoides,Elegia fistulosa, Hypodiscus aristatus, Mastersiella purpurea, Thamnocortus insignis, Wildenowia glomerata*
C. attenuatus	C2	*Cannomois primosi, Elegia neesi*	W	Hypo-Wil	*Elegia caespitosa, Mastersiella digitata, Restio parvispiculus, Wildenowia teres*
C. bicoloratus	C1	*Restio paniculatus*	R	Rest	*Restio luxurians*
C. brevipilus	C2	*Hypodiscus striatus*	W	Hypo	
C. campbelli	C1	*Thamnocortus gutherieae, Thamnochortus cinereus*	R	Tham	*Cannomois primosii, Restio calcicola, Restio gaudichaudiana, Restio parvispiculus, Restio sieberi*
C. daviesi	C1	*Restio sieberi*	R	Rest	
C. ivyae	C1	*Thamnocortus punctatus*	R	Tham	
C. linderi	C1	*Restio cederbergensis*	R	Rest	*Restio paniculatus,Mastersiella purpurea*
C. nivenus	C2	*Restio quadratus*	R	Rest	
C. pickeri	C1	*Elegia filacea*	R	Ele	
C. rawsonia	C1	*Staberoha cernua*	R	Stab	*Anthocortus laxiflorus,Restio capensis,Staberoha aemula,Staberoha vaginata*

C. shortnose	C2	*Elegia elephantina*	R	Ele	
C. turneri	C1	*Anthocortus crinalis*	W	Antho	
C. uncinatus	C2	*Mastersiella spathulata, Wildenowia incurvata*	W	Hypo-Wil	
D. capensis	D	*Restio curviramis*	R	Rest	*Anthocortus crinalis,Staberoha cernua*
D. twanella	D	*Restio capensis*	R	Rest	
D. sheilae	D	*Restio vimineus, Restio capensis*	R	Rest	

Table 3.2 The estimation of phylogenetic conservatism of preferred host use on plant and insect trees by three methods. Pagels' λ, D and the parsimony null model test. All values in bold indicate a significant departure from the null model ($p < 0.05$ = *, $p < 0.005$ = **, $p < 0.1$ = ^). For D, the symbol 'x' indicates random evolution (D similar to 1) and the symbol '**+**' indicates phylogenetic signal (D similar to 0).

Trait	Phylogeny	λ	D	</> than null
	Cephalelini			
Restio tribe preferred		**1**** [a]	-1.1 **(+)** [a]	-
Restio clade preferred		0	--- [b]	-
Restio genus preferred		0	--- [b]	-
	Restionaceae			
host use		0	0.91 (x)	-
preference / avoidance [c]		0	1.04 (x)	-
preference by Cephalelini clade [d]		**0.66** *	--- [b]	< **

a – no analysis of D, D cannot be calculated for non-binary traits

b – two seperate analyses using both preferred host tribes of *C. shortnose* gave highly similar results

c – measure of Restionaceae hosts as preferred/non-preferred by Cephalelini (see Methods)

d – Cephalelini clades from phylogeny in Fig. 3.1 A

Table 3.3 Phylogenetic conservatism of individual restiohopper species' host ranges. Blombergs' K is reported for the comparison of densities of a species on the different hosts within its range. Lambda is reported as a measure of whether restio hosts within a restiohoppers host range is closely-related or not. Significant values of K and lambda are indicated with asterisks. ($p < 0.05$ = *, $p < 0.005$ = **). *C. brevipilus* is excluded from the analyses, since it only has a single host within its host range (Münkemuller *et al.*, 2012).

Restiohopper species	λ	K
C. angustatus	0.626 **	0.1 **
C. attenuatus	0	0.1
C. bicoloratus	0	0.1
C. campbelli	0	0.1
C. daviesi	0	0.0
C. ivyae	0	0.8
C. linderi	0	0.2
C. nivenus	0	0.1
C. pickeri	0	0.0
C. rawsonia	0.72*	0.2
C. shortnose	0	0.8
C. turneri	1	1.4
C. uncinatus	0	0.1
D. capensis	0	0.0
D. sheilae	0	0.0
D. twanella	0	0.0

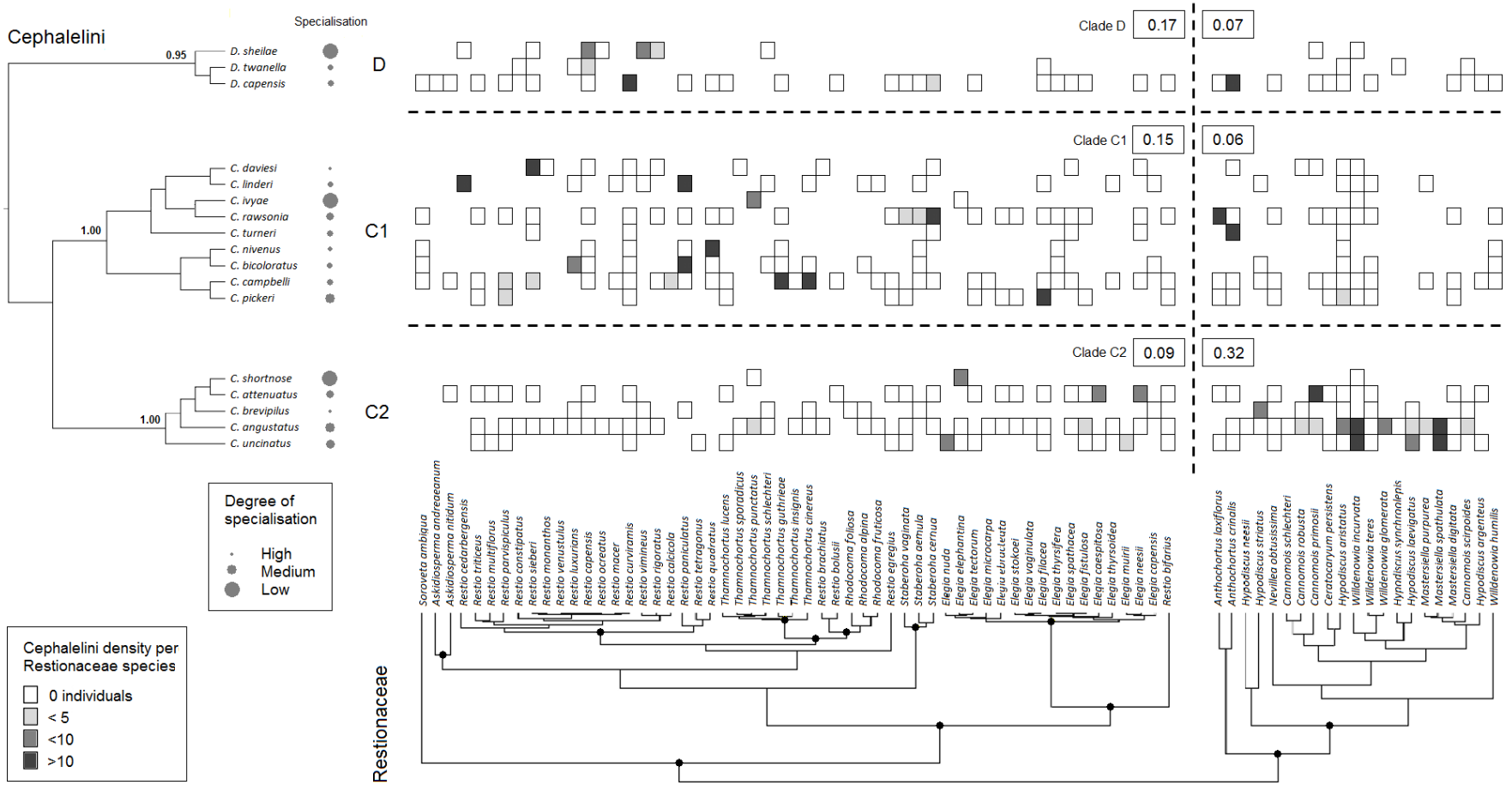

Figure 3.1 Cephalelini host range evolution. Cephalelini phylogram (top left) with circle sizes at tips indicating proportion of encountered hosts that are used, a proxy for specialisation (smaller circles = more specialised). I find no evidence of evolution towards higher or lower specialisation within the Cephalelini ($K = 0.03$, $p = 0.81$). Rows associated with Cephalelini species overlapping with columns associated with Restionaceae species (phylogram with vertical tips) indicate whether a plant species was encountered by an insect (box present) or not (box absent). Blank boxes indicate avoidance of a host while darker colours indicate increasing restiohopper abundance on a host (see legend, bottom left). Horizontal dashed lines delineate three Cephalelini clades (codes to the right of clades as in Fig. 3.1A). Vertical dashed line delineates the two Restionaceae tribes (as in Fig. 3.1A), Restioneae on the left and Wildenowieae on the right. Numbers in boxes on left and right of the vertical dashed line indicate the proportion of encountered hosts used in the Restioneae and Wildenowieae tribes respectively for each of the three insect clades. Significant difference in proportions found by Z-test for Clade C2 ($z = -3.34$, $p < 0.0005$). Sample sizes were too small to test Clade D and C1 (Sprinthall, 2002). Black dots on Restionaceae phylogeny indicate well-supported nodes (posterior probability > 0.91, Zander *et al.*, 2004).

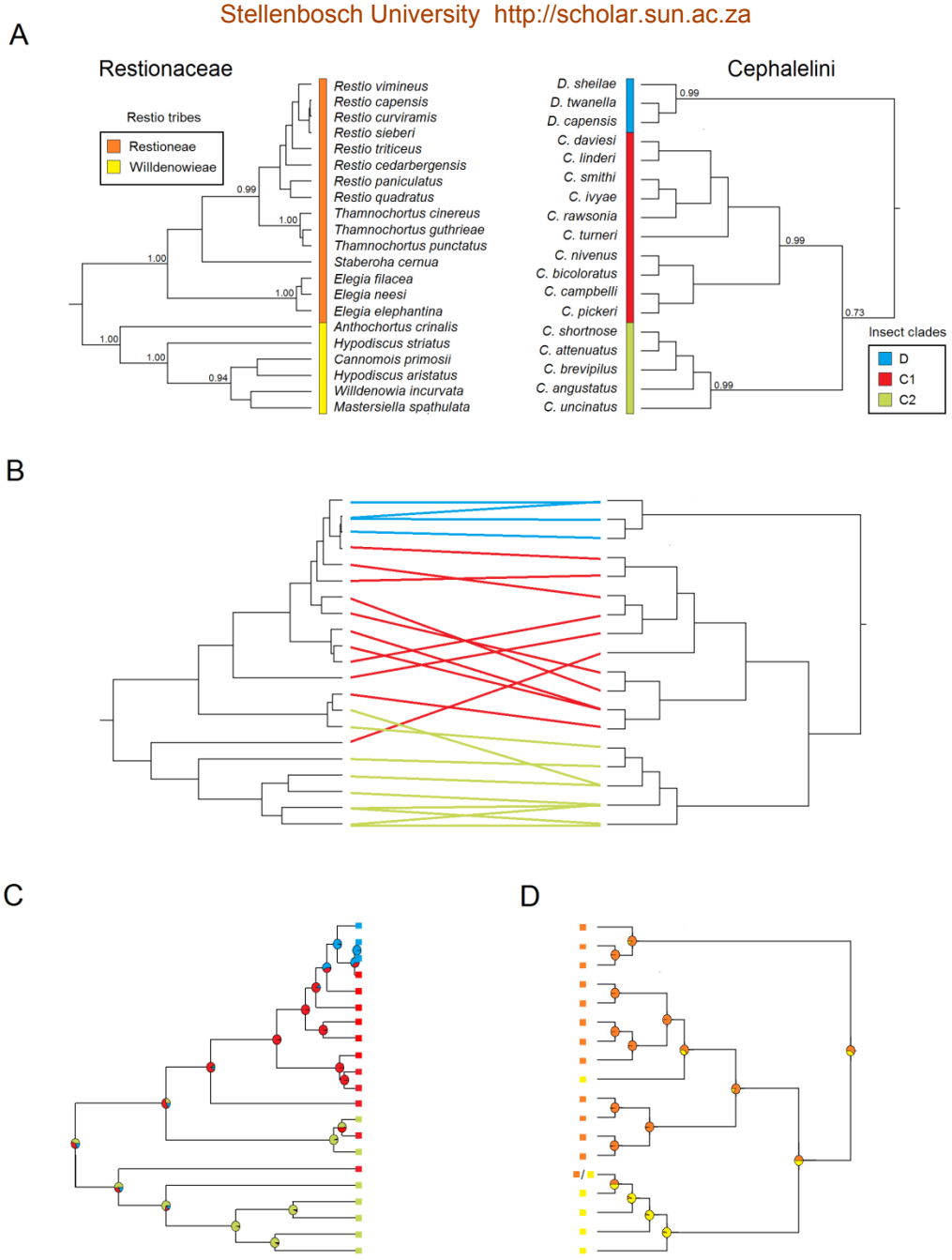

Figure 3.2 Evolution of host preference on the Restionaceae phylogeny (consistently on the left) and Cephalelini phylogeny (consistently on the right) with posterior probabilities of supported nodes indicated. **A** The phylogram on the left is a Bayesian inference phylogeny of the Restionaceae pruned to only include restio species preferred by each Cephalelini species. The colours orange and yellow at the tips of the tree indicate the Restioneae and Wildenowieae tribes (Briggs & Linder, 2009) respectively. The tree on the right is a Bayesian inference phylogeny of Cephalelini species in the genera *Duospina* and *Cephalelus*, based on *H3* and *cox1*. The colours blue, red and green indicate a *Duospina* clade, D, and two reciprocally monophyletic clades within *Cephalelus*, C1 and C2, respectively. **B** Same trees as in A with lines indicating the preferred host associations of each Cephalelini species. Line colours correspond to colours of Cephalelini clades in A. **C** Phylogram of the Restionaceae showing host preference of different Cephalelini clades (as in A), with significant phylogenetic similarity found between hosts preferred by different Cephalelini clades ($\lambda = 0.66$, $p<0.05$). **D** Cephalelin phylogram showing significant phylogenetic signal of preference for host tribes by Cephalelini species ($\lambda = 1$, D=-1.1)

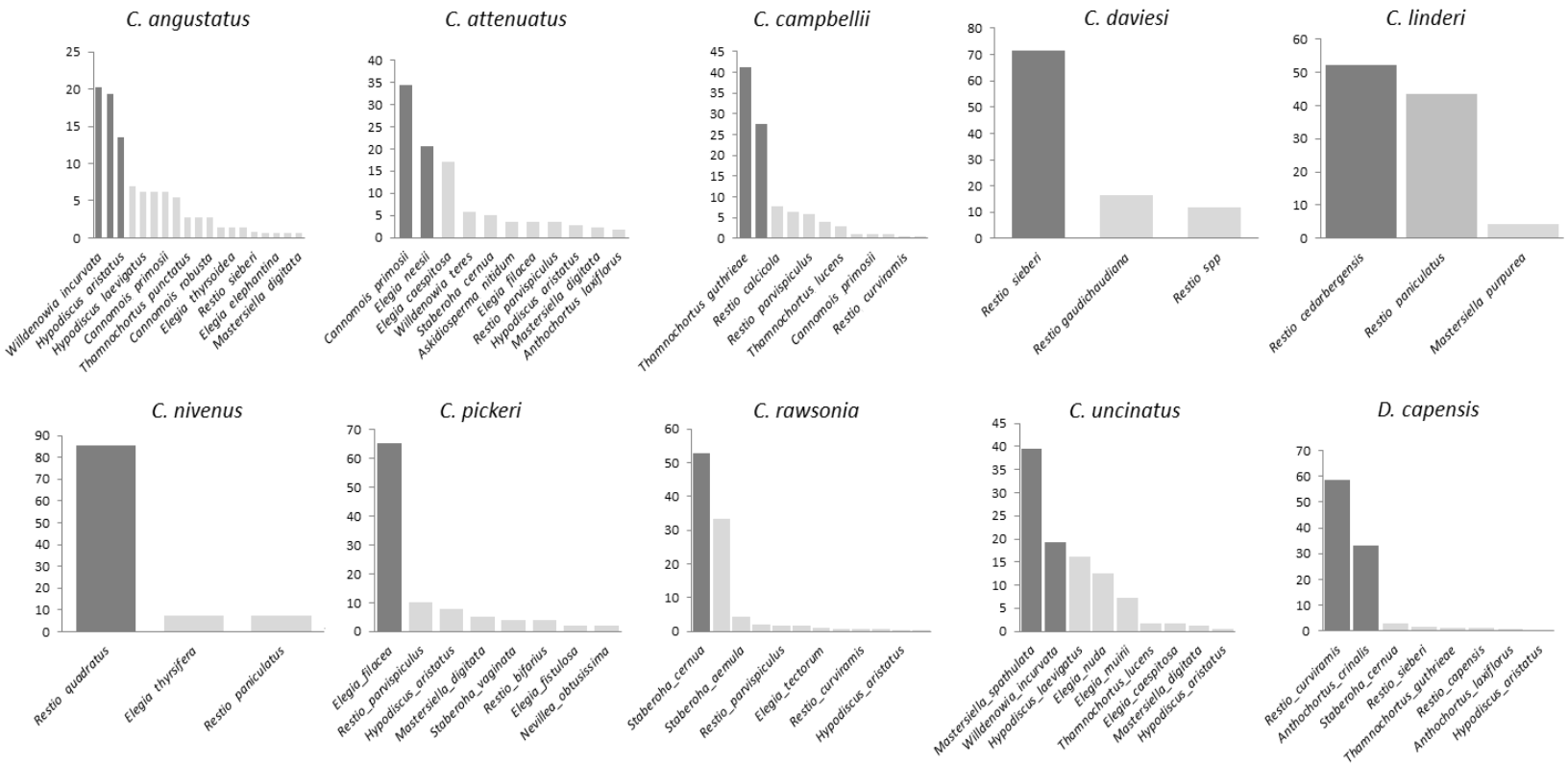

Figure 3.3 Illustration of host preference determination for 11 of the 18 species of Cephalelini. The remaining 8 had 2 or less hosts in their host range and are not presented here. Bars represent the percentage of the total abundance of a Cephalelini species added by considering densities on each additional host. Hosts are ranked from those on which a Cephalelini species is most abundant to least abundant. Dark grey bars indicate the preferred hosts that account for the first 50% of the cumulative density.

Thesis conclusion

My study of Cephalelini specialising on Restionaceae in the CFR shows that South African and Australian Cephalelini have diverged very recently (5.3-11.4 MYA), implying that the group has undergone long-distance dispersal between the continents. Their youth contradicts current thinking regarding the age of endemic invertebrates in the fynbos, which are thought to be ancient. The youth of Cephalelini also means that they could not have coevolved with their Restionaceae hosts, but insect evolution has tracked the evolution of their hosts, indicating a link between plant diversity and insect diversification for this fynbos group.

The young age of the split between Australian and South African Cephalelini identifies an avenue of faunal assemblage previously unknown in the Cape Floristic Region. Although intercontinental dispersal has been identified in two of the most representative families of the fynbos, the Proteaceae and the Restionaceae, the Cephalelini represent the first known case of animal taxa being exchanged between the fynbos biome and a biome on another continent. This recent event suggests a hitherto unconsidered potential effect on the accumulation of insect diversity in the CFR and indicates that exchanges could be ongoing. Additionally, the age of the dispersal of Cephalelini between the two continents is younger than that found for any plant taxa thus far, indicating that plant and insect dispersal into the CFR from other biomes do not necessarily occur together and might occur under different circumstances.

That the Cephalelini have not coevolved with their Restionaceae hosts is not necessarily surprising, considering that the majority of studies on plant-insect coevolution find evolutionary pressures to be asymmetrical, as I found here. Nevertheless, the Cephalelini are highly specialised in their host use, using only a fraction of the available Restionaceae hosts. Despite not having coevolved, Cephalelini evolution has tracked Restionaceae evolution, with insects having diverged in terms of their preference for different tribes of host. Cephalelini host range has diverged along the same lines, indicating strong tracking of host plant phylogeny by the insects. Contrary to what is known of the evolution of herbivorous insects, I find no indication of evolution towards increased specialisation within Cephalelini, which is probably a function of the youth of the Cephalelini occupation of Restionaceae and may well change over time.

The lack of molecular support for the monophyly of described species is unlikely to be an artifact of sample size or the gene region sampled when taking into account the young age of clades containing multiple species (<1.5MY, 5-9 species), nor do these clades only represent a single species each,

considering that described species are ecologically diverged in terms of specialist host use. Deeper nodes in the tree are likely to obtain better resolution with increased gene sampling of the additional species used and would be useful to determine whether current polytomies are real or not as well as establishing the monophyly (or otherwise) of South African Cephalelini. The fact that three species new to science were discovered during sampling while I was unable to collect all of the described species indicates that a substantial amount of Cephalelini diversity might still be waiting to be discovered. The presence of *Sulcia* bacteria within Cephalelini has proven useful in obtaining an independent source of dating information and further confirms the pervasiveness of these symbionts in the evolution of sap-feeding insects.

The most important conclusion to be drawn from this study is that research into insect evolution in the fynbos still has much to reveal about Cape diversity. The indication I provide of a recent evolutionary link between South African and Australian insect faunas is a novel finding in the study of factors influencing fynbos insect diversity. The recent diversification of Cephalelini points toward different drivers of plant and insect diversity when comparing Cephalelini to CFR plants, and also shows that diverse and highly specialised associations can arise in a relatively short amount of time. While this study indicates that insects have not been instrumental in the diversification of plants, I do find plant diversity to be an important factor in the diversification of insects. This indicates that the specialist insect fauna associated with the incredible diversity of plants in the CFR should be studied further in terms of their coevolutionary history with their hosts to determine how plant diversity affects insect diversity in other groups.

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Appendix

Figure S2.1 Chronogram of Cephalelini (top clade, in red) and the outgroups used for dating (bottom clade, in blue) based on *CO1*. Median age estimates from Bayesian inference using a priori rate calibration of *CO1* are indicated at nodes.

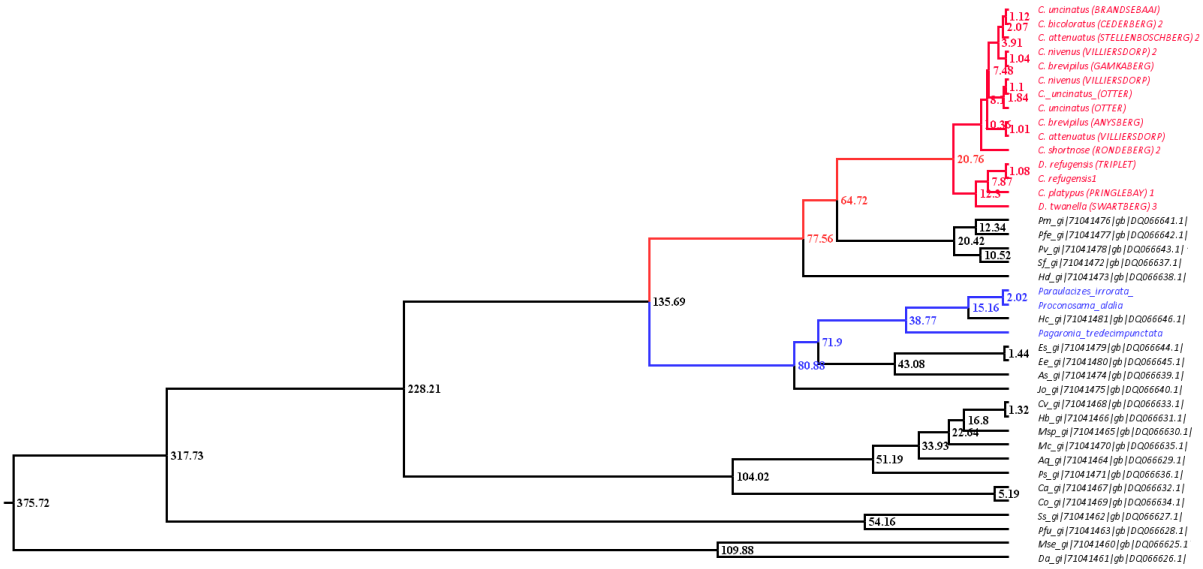

Figure S2.2 Chronogram of Cephalelini (in red) and the outgroups used for dating (in blue) based on *Sulcia* 16S rDNA. Median age estimates from Bayesian inference using *Sulcia* host fossil calibrations from Moran *et al.* (2005) are indicated at nodes.